Non-anticoagulant Derivatives of Heparin for the Management of Asthma: Distant Dream or Close Reality?

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by

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Declaration

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Given that this thesis is presented as a series of papers, either published, in press or submitted, statement of co-authorship are provided for each chapter. Due to this thesis format some repetition is inevitable.

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List of Abbreviations

AFXa	Anti-factor Xa
ASM	Airway smooth muscle
AT	Antithrombin
CAD	Charged aerosol detector
CE	Capillary electrophoresis
Con	Concanavalin
COPD	Chronic obstructive pulmonary disease
D_2O	Deuterium oxide
Da	Dalton
DMSO	Dimethyl sulfoxide
dp	Degree of polymerisation
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ECP	Eosinophil cation protein
EP	Eosinophil peroxidase
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FEV1	Forced expiratory volume
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
HPLC	High-performance liquid chromatography
HP-SEC	High-performance size-exclusion chromatography
ICAM	Intercellular adhesion molecule
IC	Ion chromatography

List of Abbreviations Continued…

IC	Ion-exchange chromatography
Ig	Immunoglobulin
IL	Interleukin
IU	International unit
KOH	Potassium hydroxide
LDH	lactate dehydrogenase
LMWH	low-molecular-weight heparin
LPS	Lipopolysaccharide
Mac	Macrophage
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
MS	Mass spectrometry
NaCl	Sodium chloride
NEDD	*N*-(1-Naphthyl)ethylenediaminedihydrochloride
NMR	Nuclear magnetic resonance
NF-κB	Nuclear factor-κB
NO	Nitric oxide
ODSH	O-desulphated heparin
PAR	Protease activated receptor
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PF	Platelet factor
PHA	Phytohaemagglutinin

List of Abbreviations Continued…

PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulated on activation, normal T cell expressed and secreted
RPIP-HPLC	Reversed-phase ion-pair high-performance liquid chromatography
RSD	Relative standard deviation
SD	Standard deviation
SNAC	Sodium *N*-[8(-2-hydroxybenzoyl)amino]caprylate
STD-NMR	Saturation Transfer Difference-nuclear magnetic resonance spectroscopy
SULF	Sulfanilamide
Th	T-helper
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-alpha
UC	Ulcerative colitis
UFH	Unfractionated heparin
UV	Ultraviolet

Abstract

Introduction

Unfractionated heparin (UFH) belongs to a class of linear, highly sulfated, acidic polysaccharides known as glycosaminoglycans. Low-molecular-weight heparins (LMWHs) are modified heparin fragments with a molecular weight range of 2000 to 8000 Da. LMWHs are prepared by either chemical or enzymatic depolymerisation of UFH. In clinical practice, UFH is largely replaced by LMWHs because of their improved pharmacokinetic profiles as decreased risk of side effects. Heparins (UFH and LMWHs) are comprised of heterogeneous mixture of anticoagulant and non-anticoagulant fractions. Much recently, these polysaccharides have shown to possess anti-inflammatory effects and such effects are strongly influenced by degree of sulfation, distribution of sulfate groups and chain length. Interestingly, the potential anti-inflammatory activities of heparins, such seen in asthma, are reported to be because of the interactions between their non-anticoagulant molecules and various biological molecules. Numerous studies highlight the role of heparins in the management of inflammatory disorders including asthma. Asthma is a common chronic inflammatory condition of the conducting airways. It is estimated that approximately 300 million people worldwide suffer from asthma and it is associated with severe morbidity, and sometimes even mortality. Asthma is known to be driven by various inflammatory cytokines including interleukin-(IL)-4, IL-5, IL-6, IL-8, IL-13 and tumor necrosis factor-alpha (TNF-α). The current drug modalities used for the management of asthma are reported to have a number of drawbacks and it is also estimated that up to 10% asthmatic patients have difficult-to-treat asthma that is often resistant to first line treatment with inhaled corticosteroids. Some recent *in vivo*

studies have confirmed that heparins possess significant anti-asthmatic activity. However, the use of heparins for the management of asthma is hindered by the risk of bleeding associated with their anticoagulant fractions. For example, in one study, administration of LMWH resulted in massive haemorrhage in a patient with inflammation. Apart from this, the investigation of anti-inflammatory effects of LMWH is challenging because these activities are expressed with high LMWH concentrations where the anticoagulant effects predominate. Therefore, the overall aim of the study was to confirm that a LMWH (e.g. enoxaparin and dalteparin) can inhibit various inflammatory mediators involved in the pathogenesis of asthma with a view of identifying non-anticoagulant fraction(s) of the parent LMWH responsible for its anti-asthmatic effect.

Methodology

Stimulation of Cells: Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of asthmatic subjects and were cultured for 72 hours in the presence of phytohaemagglutinin for the release of IL-4, IL-5, IL-13 and TNF-α. Similarly, human respiratory pulmonary epithelial cells were cultured for 24 hours in the presence of trypsin for the release of IL-6 and IL-8.

Identification of a LMWH exhibiting high anti-inflammatory activity: PBMCs were pre-treated in the presence of intact enoxaparin or dalteparin to investigate the effect of parent LMWHs to inhibit the release of tested cytokines.

Separation, isolation and desalting of parent LMWH exhibiting high anti-inflammatory effects:

Chromatographic separation of enoxaparin was carried out using ion-exchange chromatography (IC). Separations were performed on a semi-preparative CarboPac PA100 column (250 × 9 mm ID). The optimised sodium chloride (NaCl) eluent gradient was: 0-70 min: gradient from 32-74% 2 M NaCl in Milli-Q water (0.64-1.48 M NaCl). Total flow rate of 2.0 mL/min was maintained and UV detection at 232 nm was performed. The collected fractions were concentrated on a centrifugal concentrator and desalted using PD MidiTrap G-10 columns.

Determination of average molecular weight and anticoagulant activity of separated fractions:

The average molecular weight of separated fractions was determined using a Superdex^{TM} peptide 10/300 GL (300×10 mm) size-exclusion column. Isocratic elution of intact LMWH parent LMWH and the separated fractions along with the known LMWH standards were performed with a 0.3 M sodium sulfate eluent at a flow rate of 0.2 mL/min and UV detection at 232 nm was performed. The anticoagulant effect of the separated fractions was determined using low-volume microtitre plate anti-factor Xa assay using anti-thrombin III and factor Xa.

Identification of fraction(s) exhibiting high anti-inflammatory effects:

Each separated fraction of the parent LMWH was analysed for its ability to inhibit the release of tested cytokines to identify the fraction(s) responsible for the observed anti-inflammatory effect exhibited by the parent molecule.

Structural identification of fraction(s) exhibiting high anti-inflammatory effects:

The particular structure of fractions exhibiting high anti-inflammatory effect was determined using nuclear magnetic resonance. Structural characterisation of fraction(s) was performed using 1D and 2D ^{1}H spectroscopy (TOCSY 120 ms, COSY, ROESY 500 ms) and 2D ^{13}C-^{1}H spectroscopy (HSQC, HSQCTOCSY 120ms) with standard Bruker pulseprograms.

The role of sulfate groups on the observed inhibitory effect:

The parent LMWH as well as its separated fractions was selectively desulfated using well established and validated methods to investigate the character of sulfate groups which could play a role in inhibiting the release of tested cytokines. Selective desulfation at was 2-*O*, 6-*O* and *N*-sulfated positions were performed using sodium hydroxide, tetrahydrofuran and *N*-methyl-*N*-(trimethylsilyl) trifluroacetamide respectively.

Results and Discussion

When compared, enoxaparin inhibited the release of IL-4, IL-5, IL-13 and TNF-α whereas dalteparin stimulated their release. However, the clinical use of enoxaparin as an anti-inflammatory agent is largely limited because of the presence of anticoagulant fractions, being associated with the risk of bleeding. The high negative charge, structural complexity and polydispersity of enoxaparin present a special set of difficulties for its separation. Nevertheless, a novel ion-exchange chromatographic (IC) technique was developed to separate enoxaparin into several different fractions. The technique successfully resolved enoxaparin into 30 different peaks and had superior resolution than previously reported methods for the separation and

identification of various fractions of enoxaparin. The separated fractions were found to have molecular weights ranging from 600 to 8000 Da having no, low, moderate or high anticoagulant activities. Two fractions of enoxaparin were structurally identified using nuclear magnetic resonance consisting of two and four sugar units (a di- and a tetrasaccharide; approximate molecular weight 600 and 1200 Da respectively) exhibiting 85% of the inhibitory effect on the release of IL-4, IL-5, IL-13 and TNF-α displayed by the parent enoxaparin. The finding from the current study also suggests an interaction of 6-*O*-sulfated tetrasaccharide with specific or allosteric binding to cellular receptor(s) for the observed inhibition.

Parent enoxaparin was also found to inhibit the release of IL-6 and IL-8 released from human respiratory pulmonary epithelial cells. IC-derived disaccharide fraction of enoxaparin displayed the maximum inhibitory effect (>90%) exhibited by the parent molecule. Our findings indicate that the observed inhibition of IL-6 and IL-8 was most likely due to an interaction between the N-terminal domain of protease activated receptors and 6-*O*-sulfated disaccharide.

It is known that a minimum of five saccharide chain length with specific sulfation pattern is required for the anticoagulant activity of any type of LMWH. The identified fractions a di- and a tetrasaccharide (two and four sugar units respectively) exhibited high anti-inflammatory activity. This finding is important because these fractions have no effect on blood coagulation and hence, support the hypothesis that non-anticoagulant fractions of heparins are associated with anti-inflammatory.

Conclusion and Future Directions

In conclusion, a widely used LMWH (enoxaparin) was identified having high anti-inflammatory potential in inhibiting the release of critical inflammatory cytokines involved in the pathogenesis of asthma. A novel IC method was developed to separate various fragments of the parent anti-inflammatory LMWH. The method was successfully applied to investigate and identify the fractions responsible for the anti-inflammatory effects observed by the parent molecule in inhibiting the release of tested cytokines. Separated fractions of enoxaparin were structurally identified which displaying high anti-inflammatory effect. The fractions were found to have no effect on blood coagulation and therefore eliminate the potential risk of bleeding. These studies provide a solid platform for future experimental and clinical studies for the interpretation of fine/specific non-anticoagulant sequences and their precise structural information to enable better understanding of structure-activity relationships. This would allow us to answer various biological questions and to provide new targets for therapeutic interventions in different inflammatory disease states. Lastly, to identify the precise underlying cellular mechanism(s) by which the non-anticoagulant fractions of enoxaparin inhibit the release of various inflammatory cytokines associated with the pathogenesis of asthma.

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CHAPTER ONE

Non-Anticoagulant Derivatives of Heparin for the Management of Asthma: Distant Dream or Close Reality?

1.1 ABSTRACT

Background: Approximately 300 million people worldwide are currently affected by asthma. An improved understanding of the mechanisms involved in such inflammatory airway disorders has led to the recognition of new therapeutic approaches. Heparin, a widely used anticoagulant, has been shown to be beneficial in the management of asthma. It belongs to the family of highly sulfated polysaccharides referred to as glycosaminoglycans, containing a heterogeneous mixture of both anticoagulant and non-anticoagulant polysaccharides. Experimental finding suggest that heparin has potential anti-asthmatic properties due to the ability of its non-anticoagulant oligosaccharides to bind and modulate the activity of a wide range of biological molecules involved in the inflammatory process.

Areas Covered: This review focuses on the potential mechanisms of action and clinical application of heparin as an anti-inflammatory agent for the management of asthma.

Expert Opinion: Heparin may play a significant role in the management of asthma. However, these properties are often hindered by the presence of anticoagulant oligosaccharides, which possess a significant risk of bleeding. Therefore, its therapeutic potential must be explored using well-designed clinical studies that focus on identifying and isolating the anti-inflammatory oligosaccharides of heparin, and further elucidating the structure and mechanisms of actions of these non-anticoagulant oligosaccharides.

1.2 Heparin and its low molecular weight derivatives

Heparin was discovered in 1916 by Jay McLean, a medical student at Johns Hopkins Medical School, who was initially examining procoagulant phosphatides extracted from canine liver and serendipitously discovered that a compound exhibited anticoagulant effects *in vitro*; this was later named heparin [1]. Heparin belongs to the family of glycosaminoglycans (GAGs), which contains a complex heterogeneous mixture of linear, polysulfated polysaccharides composed of alternating disaccharide units of D-glucosamine and uronic acid residues linked by 1→4 glycosidic bonds (Figure 1.1) [2]. It is a naturally occurring endogenous substance, which is sequestered within the mast cells of several mammalian tissues, including the intestine, lung, liver and skin [3]. The length of heparin polysaccharide unit not only depends on its source of isolation (bovine lung or porcine intestinal mucosa) but also varies in the mast cell granules and therefore, the molecular weight composition of heparin differs [4].

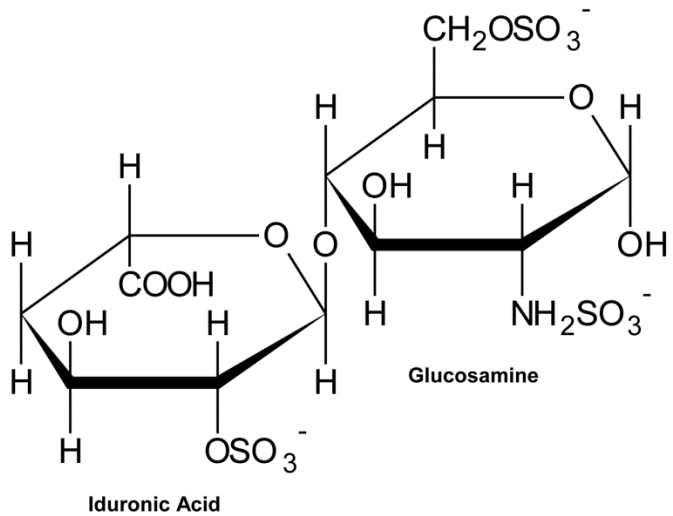

Figure 1.1 Building blocks of heparin-repeating disaccharide unit made up of alternating iduronic and glucosamine residues.

The precise mechanism by which heparin exerts its characteristic anticoagulant effect was elucidated 50 years after its discovery. It binds to serine protease inhibitor antithrombin III using its anticoagulant oligosaccharides, composed of a unique pentasaccharide sequence (Figure 1.2), thereby accelerating the inhibition of the final two proteases of the coagulation cascade (thrombin and factor Xa) [5].

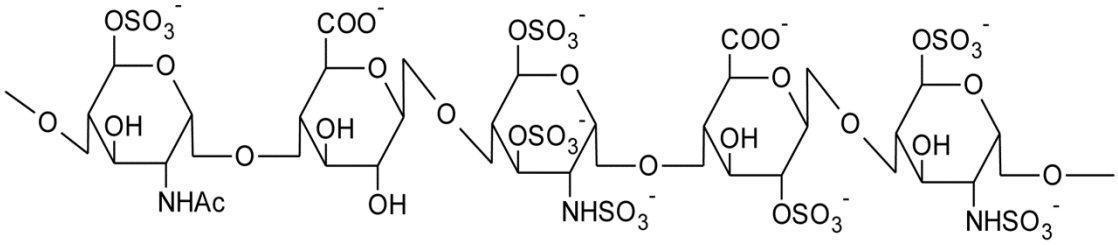

Figure 1.2 Unique pentasaccharide sequence of heparin having three glucosamine and two iduronic acid. The binding between this specific sequence and antithrombin III results in the anticoagulant effect of heparin.

Over the past 50 years, heparin has gained a widespread popularity and has been extensively used in clinical practice for its well-known anticoagulant effect. Nevertheless, heparin has been largely replaced in recent times by its clinically useful analogues known as low-molecular-weight heparins (LMWHs). The LMWHs have been proven to be more efficacious than heparin in some clinical situations, with superior pharmacokinetic profiles and reduced adverse outcomes, including heparin-induced thrombocytopaenia, bleeding, osteoporosis and the propensity to interact with a wide range of plasma proteins [6]. Currently, there are a number of commercially available LMWHs preparations which are synthesised from unfractionated heparin by either chemical or enzymatic depolymerisation processes (Table 1.1). These structurally similar analogues of heparin are also composed of anticoagulant and non-anticoagulant polysaccharides [7]. The LMWH preparations have been primarily used

for the treatment and prophylaxis of deep-vein thrombosis; they are also effective in artery bypass grafting, treatment of pulmonary embolism and maintaining vessel patency during haemodialysis [8, 9].

Table 1.1 Currently available LMWHs and their properties

LMWH	Manufacturing Process	Average Molecular Weight (Da)
Nadroparin	Deaminative cleavage with nitrous acid	4300
Reviparin	Deaminative cleavage with nitrous acid	4400
Enoxaparin	Alkaline β-eliminative cleavage of the benzyl ester of heparin	4500
Parnaparin	Oxidative depolymerisation with cupric acid and hydrogen peroxide	5000
Certoparin	Deaminative cleavage with isoamyl nitrite	5400
Ardeparin	Oxidative depolymerisation with hydrogen peroxide	5,500-6,500
Dalteparin	Deaminative cleavage with nitrous acid	6000
Tinzaparin	β-eliminative cleavage by the heparinase enzyme	6500

1.3 Heparin and inflammation

Currently, heparin and LMWHs are at the forefront in the field of glycobiology because of the recognition of various biological applications of these macromolecules beyond their well-established anticoagulant activity. Only a sub-population of heparin chains constitute a pentasaccharide sequence (anticoagulant oligosaccharide) for anti-thrombin III binding site and the majority of the chains are composed of relatively non-specific sequences (non-anticoagulant oligosaccharides) [10]. The diverse nature of heparin with respect to its molecular weight composition and sulphation pattern results in wide-ranging biological effects (specific and non-specific) [11]. The

heterogeneity of heparin enables it to interact with basic amino acids presents in numerous proteins and biological molecules, and thereby play a pivotal role in modulating inflammatory responses. The interaction of heparin with proteins is mostly non-specific and dependent on the charged groups especially the sulfate groups, charge density and the degree of polymerisation (molecular weight of an oligosaccharide chain) [12]. These interactions induce functionally important conformational changes, resulting in either aggregation of proteins to accelerate their interactions with specific cell receptors or the displacement and subsequent binding of proteins to specific cell receptors.

Heparin is thought to be an inhibitor of inflammation because of its interaction with important proteins involved in the complement system and process of inflammation, hence limiting cellular activation and subsequent tissue damage and remodelling [13]. These proteins include cytokines, growth factors, adhesion molecules, tissue-destructive enzymes and cytotoxic mediators. Specifically, heparin binds to numerous adhesion proteins present on the extracellular matrix, such as fibronectin, laminin, thrombospodin and vitronectin, and consequently alters the reactions involved in cell signalling and cell adhesion [3]. Because of the myriad of interactions with numerous proteins, heparin has been subject to intensive clinical and non-clinical investigations for potential therapeutic roles other than the control of coagulation.

Heparin has greater biological activity compared to the structurally similar members of the GAG family, like heparan sulphate. This is due to the fact that heparin is more sulphated and contains a far higher percentage of iduronate residues (Figure 1.3). The conformational flexibility of the polysaccharide chain in heparin increases

because of the presence of these iduronate residues; this flexibility, together with other electrostatic interactions, enables heparin to possess greater biological activity compared to glucuronate-containing GAGs [14]. Also, the difference in the composition and extent of sulphation of the polysaccharide chain of heparin is a key characteristic of its higher biological response. The sulphation pattern along the heparin chain is different, having sites of sulphation at the 2-*O* position of iduronate residues, glucosamine residues and glucuronic acid; 6-*O* position of glucosamine residues; and 3-*O* position of disulphated glucosamine residues. However, sulphation at the 2-*O* position of glucuronic acid and 3-*O* position of disulphated glucosamine residues is infrequent [15]. Heparin interacts with a diverse range of biological molecules; many of these molecules have preferential binding in accordance with the specific sulphation pattern within heparin. For instance, it has been elucidated that presence of sulphate groups at the 2-*O* position are required for the interaction between heparin and fibroblast growth factor (FGF)-2, while sulphation at 2-*O* and 6-*O* positions is key for its interaction with FGF-1 [16]. Similarly, for specific interaction of heparin with vascular endothelial growth factor, N- and 6-*O*-sulphate groups are essential [17]. Therefore, it can be postulated that since heparin is enriched in sulphate groups at different positions this facilitates its interaction with a wide array of biological molecules and thus enables significant biological activity compared to the other members of the GAG family.

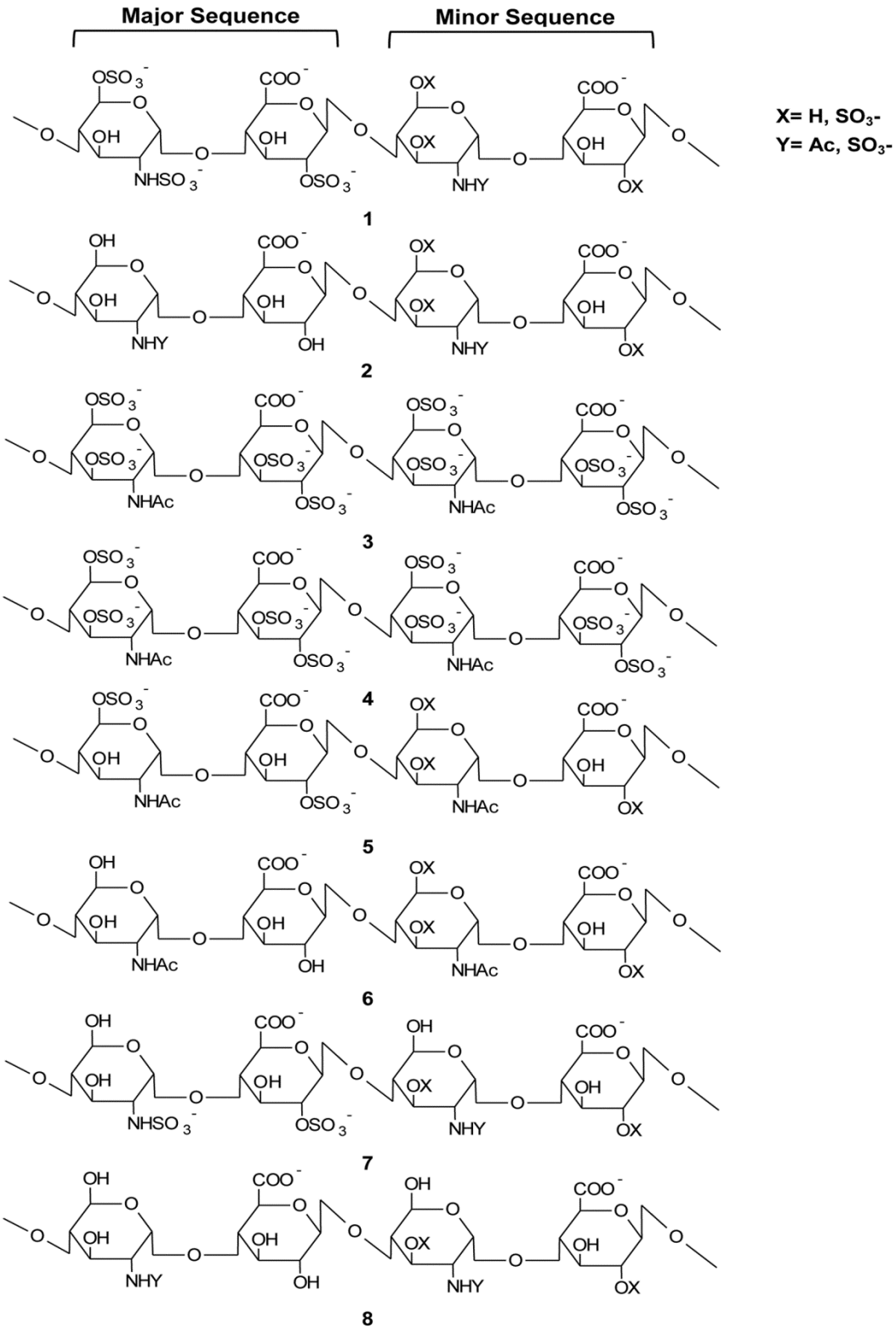

Figure 1.3 Alterations in the sulfation patterns of the repeating major and variable disaccharide units of heparin and heparan sulfate. **1**, heparin (HP); **2**, heparan sulfate (HS); **3**, fully *O*-sulfonated and re-*N*-acetylated HP; **4**, fully *O*-sulfonated and re-*N*-acetylated HS; **5**, de-*N*-sulfonated and re-*N*-acetylated HP; **6**, de-*N*-sulfonated and re-*N*-acetylated HS; **7**, 6-*O*-desulfonated HP; **8**, 6-*O*-desulfonated HS.

1.4 Asthma: an overview

Asthma is a complex multifactorial disorder of conducting airways, characterised by airway hyper-responsiveness and recurrent reversible airway obstruction [18]. The defining features of asthma that lead to decreased lung function include contraction or hypertrophy of airway smooth muscle, resulting in bronchoconstriction, and airway inflammation to airway remodelling [19, 20]. The symptoms of asthma often include chest tightness, dyspnoea, cough and wheezing. These symptoms, together with limitation of airflow, lead to the exacerbation of underlying airway inflammation, which may cause severe morbidity, and even mortality [21].

Recent advancements in the methods for investigating airway inflammation in asthma, such as brochoalveolar lavage, endobronchial biopsy, fibro-optic bronchoscopy, exhaled breath condensate and sputum induction, has led to the recognition of the cells and mediators contributing to airway inflammation in asthma [22]. Exposure to acute respiratory infection or allergens like pollution, smoke, or stress and the subsequent allergic cascade initiate an acute immune response which leads to airway inflammation. For decades, the role of the allergic cascade in the development of airway inflammation has remained the focal point of research interest. Mast cells have been recognised as the principal effector cells of allergic reaction in asthma [23]. However, other cells that contribute to ongoing inflammation include lymphocytes, eosinophils, neutrophils, macrophages, dendritic cells and structural cells (such as fibroblasts, epithelial cells and smooth muscle cells) [24].

The allergic reaction and underlying inflammation in asthma can be sub-divided into three distinct phases: the induction phase, the early-asthmatic phase and the late-phase asthmatic reaction (Figure 1.4) [23]. Antigen presenting cells (APCs)

are distributed throughout the respiratory tract (from nasal mucosa to the lung pleura). Induction of an allergic reaction is caused by the inhaled allergens that are taken up and processed by APCs and presented to the allergen-specific T and B cells present in the lymph node [25]. APCs then activate the *naïve* T-helper (Th) cells, which further differentiate into either Th1 or Th2 cells; Th2 cells are in preponderance over Th1 cells, resulting in the production of important cytokines (such as interleukin (IL)-4, IL-5, IL-9 and IL-13) involved in the development of the features of asthma [23]. Furthermore, IL-4 and IL-13 lead to the activation of B cells and concomitant production of immunoglobulin E (IgE) [26]. The synthesised IgE antibodies bind to high-affinity IgE receptor FcεRI, which is present on the mast cell surface or on the surface of the peripheral blood basophils. On the other hand, IL-5 plays a pivotal role in the pathogenesis of respiratory inflammation by rapidly attracting circulating eosinophils to the locally affected area [27]. IL-5 also primes eosinophils and mast cells which, when activated, release significantly higher quantities of IL-5 and tumor necrosis factor-alpha (TNF-α). The early-asthmatic phase is caused by the release of preformed mediators (histamine), newly synthesised lipid mediators (prostaglandins and leukotrienes), cytokines and growth factors (TNF-α, IL-4, IL-13, vascular endothelial growth factor) upon degranulation of mast cells or basophils or upon re-exposure to allergens [28]. This phase is characterised by vascular leakage, constriction of airway smooth muscle cells, enhanced airway hyper-responsiveness, mucus production and recruitment of inflammatory cells [29].

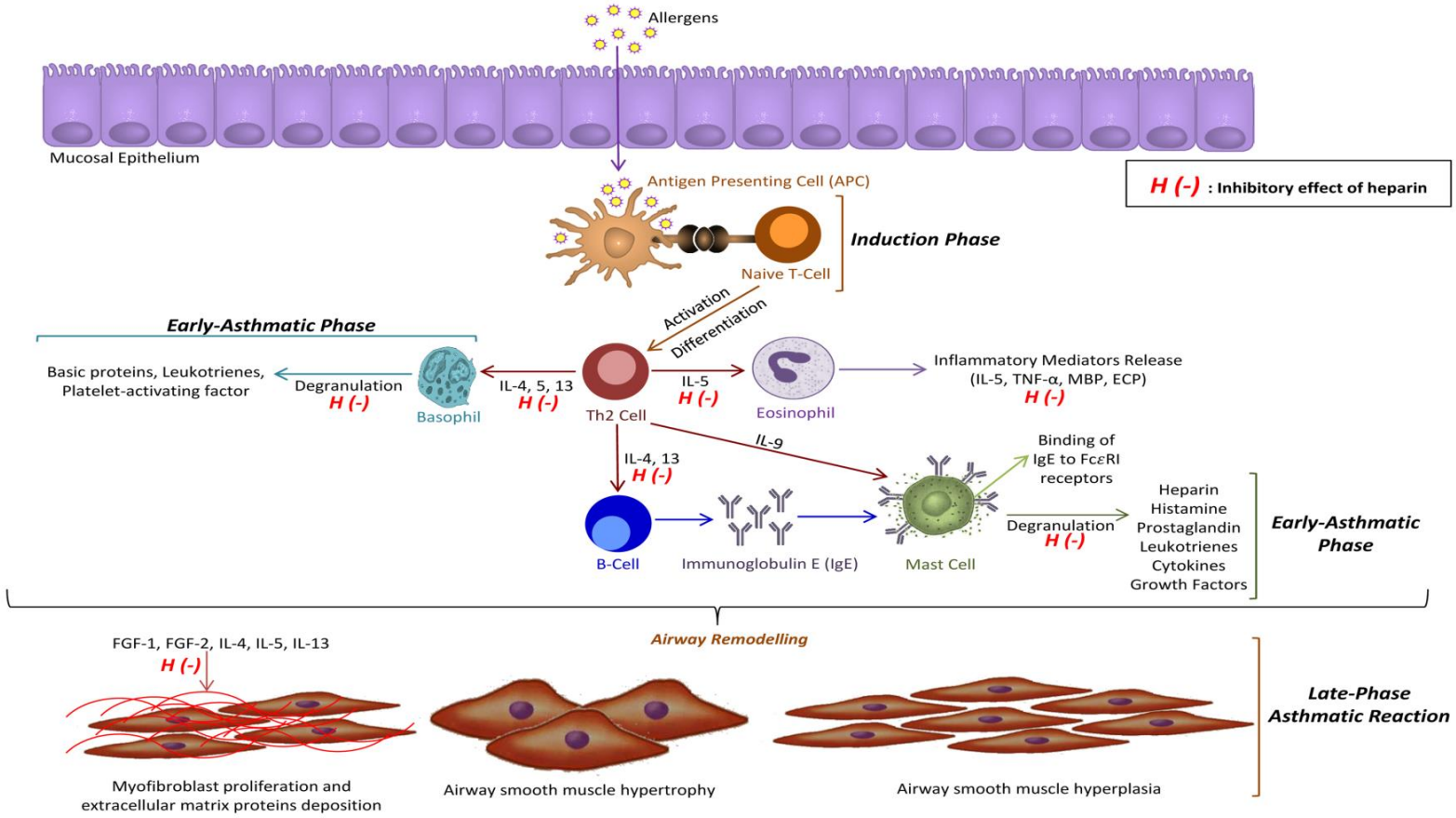

Figure 1.4 Schematic representation of the inflammatory cascade associated during distinct phases of allergic asthma. The activation and recruitment of several inflammatory cells and subsequent release of numerous inflammatory mediators that are involved in allergic airway inflammation, together with the primary structural changes that contributes to airway remodelling. H (-), denote the inhibitory effects of heparin on various inflammatory mediators shown.

Ongoing inflammation results in the late-phase asthmatic reaction, which is characterised by excessive inflammation of the airways producing permanent structural changes in the airway. The primary structural changes include the deposition of extracellular matrix (ECM) proteins, such as collagen, laminin, elastin and proteoglycans, around the airway smooth muscle (ASM), ASM hypertrophy and hyperplasia contributing to airway wall thickening, and myofibroblast proliferation associated with thickening of basement membrane (sub-basement membrane fibrosis), epithelial damage and mucus metaplasia [20, 30]. These changes are collectively referred to as airway remodelling. Several contributing factors lead to airway remodelling, these include growth factors and cytokines, such as FGF-1, FGF-2, transforming growth factor (TGF)-β_1, epidermal growth factor (EGF), IL-4, IL-5 and IL-13, which are actively involved in the enhanced deposition of ECM and ASM proliferation [31].

1.5 Biological effectiveness of heparin in asthma

It has been shown that heparin has potential in the management of inflammatory airway disorders. Heparin appears in the late foetal life and only small amounts have been detected during the early foetal development, probably because the protection against foreign bodies is not required during the early foetal life [32]. Therefore, it was suggested that, in addition to the intervention of the immune system, the manifestation of heparin also acts as one of the defense mechanisms against foreign bodies. The presence of heparin granules in the cytoplasm of mast cells and its release by inflammatory effectors, like allergens which bind to antigen-binding sites situated on mast cell surface, lead to the hypothesis that heparin is actively involved in counterbalancing the inflammatory response [33]. Heparin administration leads to

pain and swelling at the injection site; however, amelioration of pain and swelling shortly after heparin administration indicates an *in vivo* anti-inflammatory effect of heparin which has been observed in patients with deep-vein thrombosis [12]. Also, data from clinical studies indicate that elevated plasma levels of heparin and altered platelet function have been detected in atopic patients with asthma, supporting the potential role of heparin in respiratory diseases [34].

The anti-inflammatory potential of heparin has been reinforced by preclinical and clinical studies that have shown its effectiveness in the treatment of a wide array of inflammatory airway disorders, including asthma [35] and chronic obstructive pulmonary disease (COPD) [36]. It has been demonstrated that heparin binds to a large number of biologically active proteins and, thereby, leads to the inhibition of a variety of factors implicated in airway inflammation and remodelling (Figure 1.4). The consequences of these effects include the inhibition of the (i) function of T-lymphocytes [37], (ii) infiltration of eosinophils and neutrophils into lungs [38], (iii) proliferation of airway smooth muscle [39], (iv) induction of asthma by exercise [40, 41] and (v) allergen-induced early and late-asthmatic response [42]. There are many hypothesised explanations of the potential anti-inflammatory mechanisms of heparin during respiratory inflammation. The key proposed mechanisms that may account for the anti-inflammatory effects of heparin in asthma are outlined below.

1.5.1 Anionic and cationic interactions

Heparin contains a high net negative charge because of the presence of sulphate groups and therefore has a high affinity for positively charged biological proteins. Among such cationic proteins, heparin binds and inhibits eosinophil cation protein (ECP) and eosinophil peroxidase (EP) which are cytotoxic mediators released after

the activation of eosinophils [43]. ECP and EP are involved in late-asthmatic reactions causing airway remodelling. Inhibition of ECP moderates its cytotoxic effects on the respiratory epithelial cells; mucus production in the airways and ECP-mediated release of histamine and mast cells *in vitro* are repressed [44]. Other cationic proteins that are inhibited by heparin are derived from platelets such as platelet factor-4 (PF_4). The inhibition of PF_4 results in the reduction of further release of inflammatory mediators by eosinophils due to the decline in the chemotactic properties of PF_4 for eosinophils [45].

1.5.2 Anti-adhesive nature of heparin

Adherence of inflammatory cells to the vascular endothelium and their subsequent diapedesis into tissues is considered as an important element of the inflammatory response [46]. The recruitment of inflammatory cells into tissues is a multi-step process and it has been reported that heparin is active in each step to inhibit the recruitment of inflammatory cells into tissues, including the adhesion and transmigration of leucocytes to endothelial cells, and to consequently limit inflammation (Figure 1.5) [12]. The adhesion of leucocytes to the vascular endothelium is directly inhibited by non-anticoagulant derivatives of heparin [47, 48]. It was reported by Lever et. al. that the adhesion of polymorphonuclear leucocytes to endothelial cells was inhibited after pre-incubation of leucocytes with heparin [47].

Apart from this direct inhibitory action, the anti-adhesive activity of heparin is also partly due to its binding to several adhesion molecules expressed during inflammation; these include selectins (L-, P- and E-selectin) and macrophage-1 (Mac-1; CD11b/CD18) [33, 49-51]. L-selectin is constitutively expressed on the leukocytes, P-selectin on activated endothelium or platelets and E-selectin on activated

endothelium [52]. Interaction with selectin proteins and their counter glycoconjugate ligand(s) enables the leukocyte to "tether and roll" on the endothelium, which is the first step of leukocyte recruitment. Heparin acts as a counter-ligand for the selectins and binds in a calcium-dependent manner via lectin domains, resulting in the inhibition of the binding of L-, and P- selectin to their natural ligands (sialyl-Lewis^x receptors) [49]. Thus, heparin constrains the early adhesive interactions between the inflammatory cells and the vessel wall mediated by L-selectin and sequestration of neutrophils to the site of inflammation interceded by P- and E-selectin [53, 54].

The firm adhesion of leukocytes to the endothelium, another major adhesion step, is mediated by the interaction between the integrin family of adhesion molecules (Mac-1; CD11b/CD18) and intercellular adhesion molecule-1 (ICAM-1) [55]. Heparin binds to Mac-1, thus blocking the interaction between Mac-1 and ICAM-1 and, subsequently, inhibiting the adhesion of leukocytes to the endothelium [51].

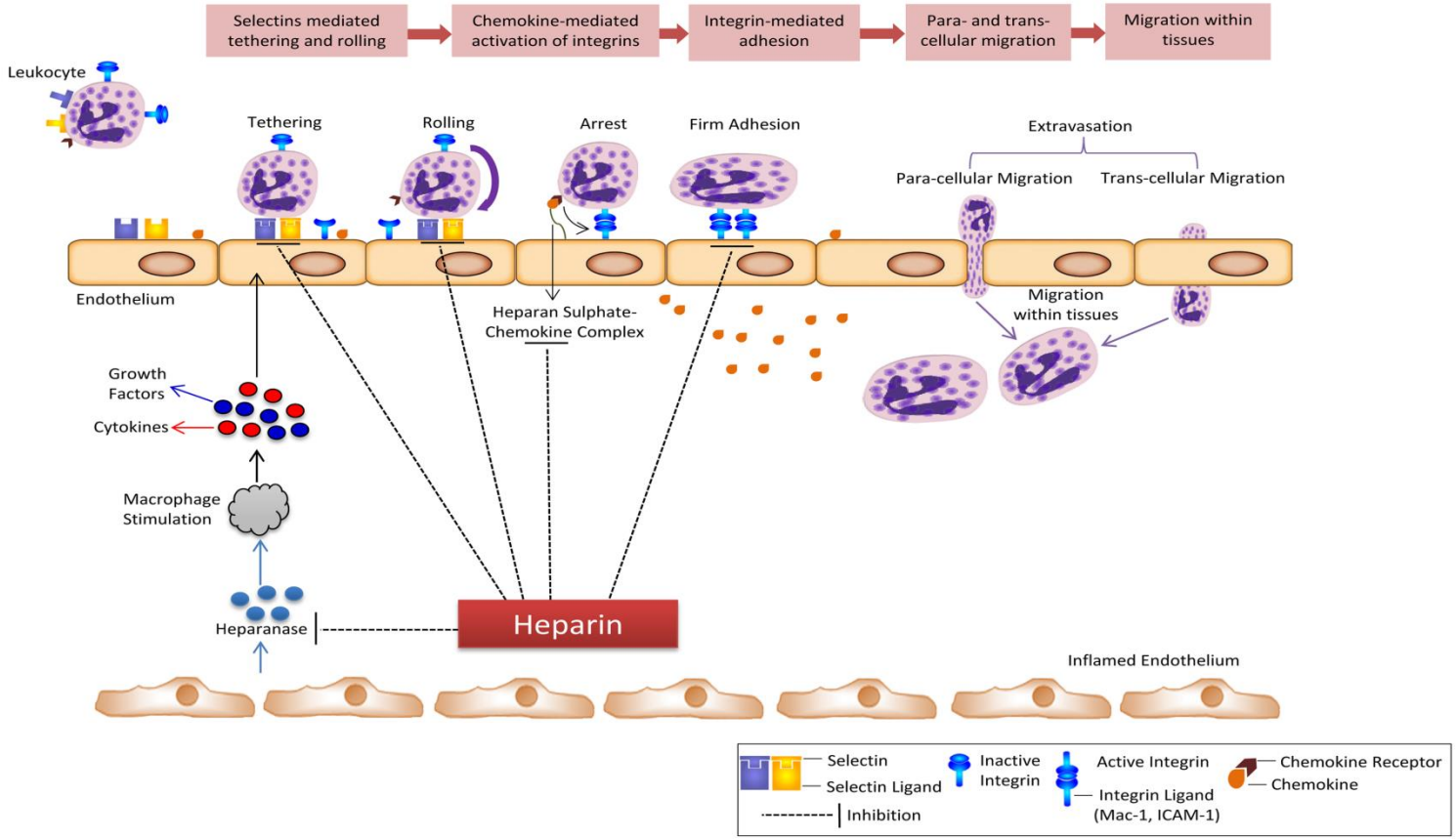

Figure 1.5 Multi-step process involving the tethering, rolling, arrest and adhesion of leukocyte along the endothelium followed by extravasation (para- or trans-cellular migration) of leukocytes into tissues and the effectiveness of heparin in limiting inflammation by inhibiting the recruitment of inflammatory cell into tissues.

1.5.3 Anti-chemokine mechanism of heparin for its anti-migratory activity

Heparin is known to neutralise or modify the properties of bound proteins, which are involved in the inflammatory process, and thereby help in limiting inflammation. Among such proteins, the migration of the leukocyte to the site of inflammation and the immune response following its activation is mediated by small, basic, chemotactic cytokines, such as chemokines [56]. It has been proposed that chemokines selectively bind to the GAGs expressed on the surface of endothelial cells and/or leukocytes, or in tissues. The interaction between chemokines and GAGs, especially heparan sulphate, results in enhanced migration of leukocytes to the site of inflammation by migration through the tissues and also by directing extravasation from the blood vessels via chemokine gradients (either chemotaxis or haptotaxis) and hence is implicated in the induction of inflammation [57].

There is strong evidence that heparin inhibits the chemotactic potential of chemokines via competitive binding to displace other GAGs such as heparan sulphate, which are bound to chemokines, forming a biologically inert or anti-inflammatory heparin-chemokine complex (Figure 1.5) [58]. The displacement of heparan sulphate also prevents the binding of chemokines to their receptors, as it is known that heparan sulphate is also involved in presenting chemokines to their receptors [59].

1.5.4 Inhibition of the endoglycosidase heparanase

Heparin acts as competitive inhibitors of heparanase, which is an endoglycosidase enzyme released from inflamed epithelial cells and a number of inflammatory cells including platelets, neutrophils, monocytes and activated T-lymphocytes [60]. The enzyme is believed to play a significant role during inflammation, causing

degradation and remodelling of extracellular macromolecules which enhance endothelial retraction and migration of leukocytes through the vascular endothelium [37, 61]. Heparanase is also involved in the direct activation of macrophages, resulting in the release of various cytokines and growth factors that promote inflammation. Heparin has the potential to modulate cell trafficking via its anti-adhesive and anti-chemokine mechanisms. In addition to these mechanisms, the inhibition of heparanase by heparin reduces cell diapedesis and trafficking into tissues (Figure 1.5).

1.5.5 Inhibition of 1,4,5-inositol triphosphate

The therapeutic utility of non-anticoagulant oligosaccharides of heparin is often hindered by the presence of anticoagulant oligosaccharides causing the risk of bleeding. Nevertheless, the use of high doses (up to 80,000 U) of heparin in aerosolised form by Ahmed et al. [40] circumvented the risk induced by anticoagulant oligosaccharides. It was observed that after the patients inhaled aerosolised heparin, the specific post-exercise airway conductance value did not decrease when compared with positive control (cromolyn sodium; a mast cell stabiliser) and heparin was shown to be effective in the prevention of exercise-induced asthma. In contrast, no change in the level of coagulation was detected by monitoring the partial-thromboplastin time. However, the mechanism via which heparin was effective in preventing a decrease in the post-exercise airway conductance value is still uncertain. Ahmed et al. postulated that the protective action of heparin on exercise-induced asthma could be due to its inhibition of second-messenger (1,4,5-inositol triphosphate) mediated calcium release, through the blockade of receptors present on lung mast cells, leading to the inhibition of signal transduction, mast cell degranulation and mediator release. However,

alongside 1,4,5-inositol triphosphate, there are multiple other factors responsible for causing exercise-induced asthma, which were not considered in the study conducted by Ahmed et al.

1.5.6 Inhibition of nuclear factor-κB (NF-κB)

NF-κB is a pivotal transcription factor which, upon activation by nuclear translocation of cytoplasmic complexes, increases the pro-inflammtory gene expression leading to the synthesis of cytokines, chemokines, adhesion molecules, growth factors and enzymes [62]. Several lines of evidence indicate that NF-κB plays a central role in inflammatory airway diseases and persistent activation of the NF-κB pathway has been observed in asthmatic tissues [63]. The persistent activation of NF-κB via a variety of stimuli (physical and chemical stress, T and B cell mitogens, lipopolysaccharides) is involved in the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, IL-12, IL-18 and IL-1β, which may influence the severity of chronic inflammatory diseases [62].

It has been recently reported that heparin exerts an anti-inflammatory response by inhibiting p38 mitogen-activated protein kinase (MAPK) and NF-κB, subsequently quenching the activation of endothelial cells induced by lipopolysaccharides [64]. A study has documented a significant decrease in the release of interferon- γ and TNF- α by activated T-lymphocytes when pretreated with sulphated disaccharides of heparin. It was recognised that this effect of heparin was due to the inhibition of NF-κB activation of anti-CD3 activated T-lymphocytes [65]. As described previously, the extent of sulphation of the polysaccharides chain of heparin is a key characteristic for its higher biological activity. Similar response was observed in this study, suggesting that the degree of sulphation is essential for the inhibition of NF-κB; tri-suphated

disaccharide exhibited an inhibitory effect on NF-κB, but not the mono- or un-sulphated disaccharides.

1.5.7 Inhibition of inflammatory mediator release

Proteoglycans, including heparin, are released after degranulation of mast cells during the early-asthmatic phase. It has been hypothesised that the physiological role of heparin in asthma could be due to its binding to, and consequent inhibition of, the preformed mediators such as histamine, leukotrienes and cytokines (IL-4, IL-5 and IL-13) released during the early-asthmatic phase. On the other hand, heparin can also inhibit the release of preformed mediators by preventing mast cell degranulation via its competitive binding to IP_3 receptors, hence controlling stimulus-secretion coupling in mast cells [66]. An alternative hypothesis by Page [67] specified that the natural anti-inflammatory mechanism of the released endogenous heparin is inhibited by the prolonged use of β_2-agonists in asthma, which reduce mast cell degranulation. Consequently, the prolonged use of adrenoceptor agonist deprives the release of heparin and other protective factors, which play a defensive role in the management of respiratory disorders.

A wide array of inflammatory mediators, including cytokines, chemokines and complement factors, are released from the inflammatory cells during asthma. Among all the inflammatory cell types, mast cells play a major role in promoting ongoing airway inflammation and occurrence of late-phase asthmatic reactions through the release of multifunctional cytokines (TNF-α, IL-3, IL-4, IL-5, IL-6, IL-8, IL-16 and GM-CSF), chemokines (macrophage inflammatory protein- MIP-1, monocyte chemotactic protein- MCP-1, regulated on activation, normal T cell expressed and secreted- RANTES) and growth factors (transforming growth factor- TGF-β,

fibroblast growth factor- FGF) [68]. Heparin has the ability to bind and neutralise the release of inflammatory mediators and thereby attenuate the ongoing inflammatory process by condensing the influx of inflammatory cells, including eosinophils, T-lymphocytes and neutrophils into the site of inflammation. Numerous clinical studies have documented that GAGs like heparin or heparin-derived oligosaccharides are able to bind and modulate the activity of several cytokines, chemokines and growth factors that are involved in the early or late asthmatic reaction and contribute to inflammatory disease severity. These include IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, TNF-α, PF_4, MIP-1α, MIP-1β, MCP-1, RANTES, platelet-derived growth factor, acidic and basic FGF, TGF-β, hepatocyte growth factor and vascular endothelial growth factor [69-80]. Additionally, heparin has been shown to bind and inhibit the activity or release of inflammatory mediators like cathepsin G [81] and elastase [82], which are toxic to bronchial epithelium and promote the ongoing inflammatory response causing tissue damage and remodelling. Furthermore, heparin interacts with secretory leukocyte protease inhibitor, through which it enhances the inhibition of cathepsin G and human neutrophil elastase [83]. The highly anionic nature of heparin enables it to bind and inhibit cytotoxic eosinophil-specific inflammatory mediators including major basic protein (MBP) and eosinophil peroxidase [84, 85]. Therefore, it can be seen that heparin exhibits a broad spectrum of anti-inflammatory activities by exerting a variety of protective mechanisms [86]. Nevertheless, the fundamentals for the management of asthma seem to be heparin-mediated modulation of an array of inflammatory mediators released from the inflammatory cells.

1.5.8 Airway remodelling

As mentioned, persistent inflammation is associated with airway remodelling which is due to the permanent structural alteration of the airways. Among several structural changes, the key alterations associated with airway remodelling are ASM cell hypertrophy and hyperplasia leading to the thickening of ASM [30]. It is noteworthy that these structural changes are generally insensitive or not fully responsive to the currently available treatments like corticosteroids [31, 87]. Heparin, however, has anti-proliferative effects and is able to inhibit the proliferation of ASM [88]. Nonetheless, the anti-proliferative activity of heparin largely depends on its molecular weight, charge density, position and degree of sulphation, as desulphated and disaccharides moiety of heparin failed to inhibit ASM proliferation [88]. The anti-proliferative activity of heparin increases with an increase in the negative charge of the polymer [89]. Alongside the negative charge, the anti-proliferative activity of heparin also requires oligosaccharide chain greater than six saccharides; for instance, the non-anticoagulant heparin decasaccharide exhibited anti-proliferative effects [90]. On the other hand, selectively *O*-sulphated heparin retained anti-proliferative activity and oversulphation at *O*-positions enhances this activity [89, 91]. Remarkably, only the non-anticoagulant oligosaccharides of heparin retained these effects, since the pentasaccharide sequence (anticoagulant oligosaccharide) specific for anti-thrombin III binding, having a 3-*O*-sulphate group, displayed negligible anti-proliferative activity [89].

Furthermore, heparin is also capable of modulating the effects of many basic growth factors that are crucially involved in airway remodelling, such as FGF-1, FGF-2 and TGF-β_1 [39, 79]. There are a few studies suggesting that heparin may inhibit the

binding of EGF to its receptor and could serve in EGF-mediated inflammation in different disease models [92, 93]. It is evident that EGF is one of the major drivers of inflammation and airway remodelling in asthma [31]. However, to date there is no clear evidence of the effectiveness of heparin in inhibiting EGF-mediated asthmatic reactions.

1.6 Clinical Studies

The anti-inflammatory potential of heparin for the treatment of acute asthma was first assessed in 1960 using intravenously administered heparin [94]. In this small, subjective trial, heparin was found to be effective by immediately ameliorating the symptoms of asthma (wheezing, cough and difficulty in breathing). Since then, a large number of randomised trials have focused on the potential efficacy of heparin and its low-molecular weight derivatives in the management of asthma (summarised in Table 1.2). In most cases, heparin was found to be effective in improving the signs of asthma - for example, the inhibition of exercise and methacholine-induced bronchoconstriction [40, 41, 95], inhibition of bronchospastic response induced by house dust mite [35, 42], and inhibition of eosinophil recruitment after nasal allergen challenge [96]. Remarkably, none of these trials reported heparin induced haemorrhagic side effects. Later a trial conducted by Bendstrup et al. [97] on lung deposition and clearance of inhaled heparin reported that after 24 h, 40% of the single-inhaled dose of heparin was detected in the lung, with no effect on blood coagulation.

Table 1.2 Clinical Studies

Trial Design	Type of Asthma	No. of Subjects	Heparin/LMWH Type/Dose Tested	Experimental Design	Delivery System	Effects	Reference
Double-blind, placebo-controlled, crossover design	Early asthmatic response	15	20,000 U heparin	Administration of heparin 10 min before antigen challenge	Nebulization	Bronchospastic response induced by house dust mite was inhibited	[35]
Randomised, single-blind, crossover design	Exercise-induced asthma	12	4 mL of 20,000 U/mL heparin	Administration of heparin sodium from 0.25 to 6 h before exercise challenge	Nebulization	Bronchoconstrictive response was inhibited from 0.25 to 3 h; ineffective at 6 h	[40]
Randomised, double-blind, crossover design	Exercise-induced asthma	13	4 mL, 7.5 mg/Kg Heparin or 0.5, 1 and 2 mg/Kg enoxaparin	Administration of heparin or enoxaparin 45 min before exercise challenge	Aerosolized	Bronchoconstrictive response was attenuated by 28%, 38% and 48% at 0.5, 1 and 2 mg/Kg enoxaparin, respectively and by 31% using heparin	[41]
Randomised, two-period, double-blind, crossover design	Early and late asthmatic response	8	1,000 U/Kg heparin	Administration of heparin sodium at 0.5 and 1.5 h before and 2, 4 and 6 h after antigen challenge	Nebulization	Immediate asthmatic response was attenuated and the late asthmatic response was significantly reduced	[42]

Randomised, single-blind, crossover design	Bronchoconstrictive response to bronchostimulants	13	1,000 U/Kg heparin	Administration of heparin sodium 45 min before provocation with methacholine	Nebulization	Methacholine-induced bronchoconstriction was inhibited	[95]
Randomised, placebo-controlled crossover design	Early and late asthmatic response	10	20,000 U heparin	Administration of heparin calcium followed by antigen challenge	Nebulization	No effect	[98]
Randomised, double-blind design	Bronchoconstrictive response to bronchostimulants	11	4 mL, 1,000 U/Kg	Administration of heparin sodium 10 min before provocation with methacholine or metabisulphite	Nebulization	No effect	[99]
Randomised, single-blind, crossover design	Exercise-induced asthma	9	4 mL of 20,000 U/mL heparin	Administration of heparin sodium from 15 min to 6 h before exercise challenge	Nebulization	Bronchoconstrictive response was inhibited from 15 min to 3 h; ineffective when administered before 6 h	[100]
Double-blind, placebo-controlled, crossover design	Early and late asthmatic response	13	5,000 U Heparin	Administration of heparin sodium 10 min before provocation with allergen	Nebulization	Allergen (grass pollen) induced airway obstruction and bronchial hyper-responsiveness was inhibited	[101]

Since inhaled heparin has shown favourable effects with no adverse changes to coagulation parameters, combined therapy with currently used standard medication could serve as a useful approach in the clinical management of asthma. For instance, one such combined therapy has already been tested in a trial with patients suffering from COPD, showing the effectiveness of enoxaparin with a combination of salmeterol/fluticasone [36]. Similar approaches could be tested in clinical trials with asthmatic subjects.

1.7 Degree of uncertainty associated with anti-inflammatory effects of heparin

Although inhaled heparin has shown encouraging results for the management of asthma, the preliminary clinical data are limited. There are several questions yet to be answered in regards to the use of inhaled heparin, including its long-term efficacy and adverse effects, dose and duration of action, before it can be recommended as an alternative or adjunct therapy for the management of asthma. Administration of heparin via oral route would be preferable over inhalation. However, very little or no gastrointestinal absorption of heparin is reported after its oral administration [102]. Nevertheless, attempts have been made to improve the absorption of oral heparin. For example, the oral absorption of heparin was found to be promoted when administered with a delivery agent such as sodium *N*-[8(-2-hydroxybenzoyl)amino]caprylate (SNAC) [103]. Lately, Ahmed et. al. [104] compared the effectiveness of oral and intravenous heparin tetrasaccharide on allergic airway responses. It was found that orally delivered heparin had comparable anti-allergic activity to that of intravenous heparin. This also suggests that orally administered tetrasaccharide had good oral bioavailability. However, the potential mechanism behind the absorption of orally administered tetrasaccharides needs to be elucidated. Other studies have indicated that

the larger oligosaccharides such as hexa- and deca-saccharides are also responsible for the anti-inflammatory activity of the parent heparin [70, 90, 105] and the oral bioavailability of such oligosaccharides is currently unknown. Nevertheless, delivery agents like SNAC could be further evaluated for oral administration of such larger oligosaccharides.

Importantly, the use of heparins as anti-inflammatory agents is handicapped not only because of the risk of bleeding associated with anticoagulant oligosaccharides but also due to inconsistent results observed in various pre-clinical and clinical studies. Despite some preliminary clinical studies showing promising outcomes with the use of inhaled heparin, other randomised clinical trials have reported conflicting results [98, 99]. For example, one of the randomised trials reported an inhibitory effect of inhaled heparin on methacholine-induced bronchoconstrictive response [95]. In contrast, no such inhibitory effect of heparin on bronchoconstriction was observed in another randomised trial [99]. Likewise, recent clinical studies investigating the anti-inflammatory potential of different LMWHs have shown varying results. For instance, tinzaparin was the most potent inhibitor of selectins among all the tested LMWHs, and enoxaparin had a greater interference with P- and L-selectin compared to nadroparin [106, 107]. The contradictory results observed with heparin preparations are not only restricted to inflammatory airway disorders, but such findings have also been reported in many other types of inflammatory conditions. For instance, several studies have reported that heparin preparations can be beneficial for the management of ulcerative colitis and lichen planus. However, there are also reports indicating heparins have no or little therapeutic effect when used for the management of the same conditions [108, 109]. One likely explanation for these inconsistent findings could be the compositional

differences between different heparin preparations. All unfractionated heparin and LMWH preparations display many similar physical, chemical and biological properties. However, a close examination has suggested significant structural differences between different LMWHs. Quantitative comparisons of the polysaccharide mixture by polyacrylamide gel electrophoresis showed substantial difference between oligosaccharide components of various LMWH preparations. Detailed study of different LMWHs by nuclear magnetic resonance showed enormous differences in their structures [110]. Two LMWHs, dalteparin sodium and nadroparin sodium, both are produced by nitrous acid treatment. Disaccharide and oligosaccharide analysis showed that they are more similar than two other LMWHs produced by different processes, but less similar than two batches of a LMWH from a single manufacturer [111, 112]. Anti-inflammatory effects of heparins are mainly attributed to their non-anticoagulant oligosaccharides and the commercially available heparins are standardised only according to the anticoagulant activity, which results in batch-to-batch variations of non-anticoagulant oligosaccharides. For example, using capillary electrophoresis, Patel et. al. [113] have demonstrated the differences in composition of non-anticoagulant oligosaccharides between batches of a LWMH obtained from the same manufacturer. They suggested that these compositional differences between non-anticoagulant oligosaccharides could be due to the inherent structural variability of the precursor heparin from which the LMWH is derived or due to differences in the fractionation process. Also, enoxaparin was reported to be effective for the management of lichen planus, only when it was derived from a selected batch, indicating not all the batches of enoxaparin preparations possess equivalent anti-inflammatory activity [109]. Therefore, a key aspect in the use of heparins as effective anti- inflammatory agents is the necessity to maintain

consistency of non-anticoagulant oligosaccharides between different batches. An appropriate way seems to be to obtain the standard electrophoretic or chromatographic separation profiles of non-anticoagulant oligosaccharides of the particular batch of heparin having an anti-inflammatory activity. Other batches of enoxaparin could be then verified for the batch-to-batch uniformity by comparing the separation patterns of their non-anticoagulant oligosaccharides against a standard separation profile. This could minimise both batch-to-batch variations of non-anticoagulant oligosaccharides and inconsistency in clinical outcomes.

1.8 Development of novel non-anticoagulant analogues of heparin

Given that the anti-inflammatory potential of heparin is independent of its anticoagulant effect, the development of novel non-anticoagulant oligosaccharides of heparin which retain the anti-inflammatory properties of the parent compound could be useful in the future for the treatment of inflammatory diseases, including asthma. An example of such preparation is 2,3-O-desulphated heparin (ODSH), which is developed by selective desulphation of unmodified heparin under extreme alkaline conditions. The ODSH preparation had no anticoagulant activity, on the other hand exhibited anti-inflammatory potential similar to that possessed by the parent heparin [91]. In pre-clinical studies, ODSH showed promising anti-inflammatory effects by inhibiting airway hyperactivity and airway smooth muscle proliferation in mammals and, therefore, it is being subjected to clinical trial in patients with exacerbations of COPD [114].

Another approach to minimise heparin-induced bleeding complications when used for the management of inflammatory disorders is to separate, isolate and characterise the non-anticoagulant and anticoagulant oligosaccharides. The current

approach to identify the non-anticoagulant oligosaccharides of heparin is to perform its depolymerisation by chemical or enzymatic methods [33]. These depolymerised oligosaccharides are then tested for their anticoagulant and anti-inflammatory effects. However, a chemical or enzymatic depolymerisation results in the structural modification of oligosaccharides and it has been demonstrated that certain biological functions of the parent heparin could indeed be removed by the depolymerisation process [115]. Some oligosaccharides in heparin are heat sensitive and can undergo chemical modification, especially desulphation during the elevated temperatures of the depolymerisation process [116]. The sulfation pattern of an oligosaccharide is a key characteristic for its anti-inflammatory properties. Depolymerisation can also be performed through a freeze-drying process; however, freeze-drying results in physical changes of some oligosaccharides present within the parent heparin molecule [117]. Therefore, the non-anticoagulant oligosaccharides obtained by chemical or enzymatic depolymerisation could have different biological effects than the parent molecule. Another approach to identify the non-anticoagulant oligosaccharides without their structural modification could be to separate and isolate the non-anticoagulant and anticoagulant oligosaccharides and examine their anti-inflammatory effects. However, separation of such a complicated molecule is a long-standing problem. Nevertheless, in recent years various separation techniques, such as capillary electrophoresis [113], reversed-phase ion-pair chromatography [118, 119] and ion-exchange chromatography (IC) [105], have been developed not only for the separation but also for the finger printing of LMWHs. For example, we have recently developed a novel IC technique capable of separating a LMWH into several different oligosaccharides with high, low and no anticoagulant activities [105]. Inhibitory effects of such IC-derived oligosaccharides on the release of macrophage-derived nitric oxide (NO) were

investigated. It is known that the production of NO in inflammatory disorders, including asthma, is increased in response to the secretion of cytokines and endogenous lipopolysaccharides [120]. It has been found that oligosaccharides with no or low anticoagulant activity significantly inhibited NO production. On the other hand, oligosaccharides having high anticoagulant effect had minimal or no effect on NO production [105]. Investigations with such approaches may lead to new formulations of purified heparin oligosaccharide (or combination of purified oligosaccharides) that exhibit effective anti-inflammatory properties but are devoid of anticoagulant activity.

1.9 CONCLUSION

In summary, the authors believe that heparin may play a significant role in the management of asthma. However, further trials on the efficacy of heparin or its derivatives are warranted before they can be indicated in the management of asthma. Its potential must be explored using well-designed clinical trials and experimental studies that also focus on identifying the anti-inflammatory oligosaccharides of heparin and further elucidating the mechanisms of action of these non-anticoagulant oligosaccharides.

1.10 EXPERT OPINION

The current drug modalities used for the management of asthma are reported to have a number of drawbacks. In general, conventional agents like salbutamol have a relatively short duration of action and associated with reduced lung function when over-used [121]. On the other hand, the safety of long-acting agents such as salmeterol has come into question, as these agents when used alone, may increase mortality or potentially result in lessened asthma control with time [122]. At present,

corticosteroids are the only effective agents available which diminish bronchial inflammation and chronic hyper-reactivity. However, inhaled corticosteroids as a first-line therapy often do not adequately control inflammatory airway disorders [123, 124]; a sufficient number of patients suffer from adverse effects and the symptoms of asthma recur within few weeks or one year after the cessation of the treatment [125, 126]. Apart from the risk of osteoporosis with long-term use, the inhaled corticosteroids are considered relatively safe in adults, while these agents are associated with significant toxicity in children, including reduced bone density and growth if used in high doses [127]. Thus, the search for safer and more effective agents for the management of asthma continues. Among these agents, heparin has attracted much research interest for its paradoxical response(s). Heparin, a commonly used anticoagulant, is a heterogeneous mixture of complicated and highly negatively charged polysaccharides. It is now well recognised that heparin possesses a wide range of biological activities, including anti-inflammatory effects. A number of studies have indicated that the anti-inflammatory effect of heparin could be due to the presence of non-anticoagulant oligosaccharides. However, these anti-inflammatory properties are largely hindered by the risk of bleeding caused by the presence of anticoagulant oligosaccharides. Therefore, there has been interest in developing pharmaceutical formulations containing non-anticoagulant oligosaccharides which are devoid of anticoagulant activity. Hence, in the future, molecular biologists and structural chemists will be focused on two existing challenges: (i) what are the underlying cellular and molecular mechanisms for the anti-inflammatory effects of these oligosaccharides? (ii) what are the differential structural requirements of oligosaccharides that possess anti-inflammatory properties? To develop a better understanding of the anti-inflammatory mechanisms of heparin-derived

oligosaccharides, a more detailed knowledge of their structures is required. There is evidence that the anti-inflammatory effects of heparin are mediated through multiple mechanisms including inhibition of macrophage-induced nitric oxide synthesis as well as inhibition of T lymphocytes-mediated release of inflammatory cytokines. We and others have demonstrated that the hexasaccharide nature of heparin is largely responsible for the inhibition of nitric oxide synthesis and the suppression of inflammatory cytokine release [70, 105]. Therefore, the key question for the molecular biologists would be whether the binding of heparin oligosaccharides to various inflammatory proteins requires unique structural sequences in the oligosaccharide chains for each protein, as with the pentasaccharide sequence found for anti-thrombin III binding, or the particular sequence allows the binding of oligosaccharides to a wide range of proteins and adhesion molecules involved in inflammation. However, given that heparin's polysaccharides have a high degree of structural diversity, their structural characterisation appears to be the biggest challenge. Most often heparin polysaccharides are structurally characterised using a bottom-up approach. In this approach, intact polysaccharides are depolymerised into smaller disaccharide fragments and then subjected to structural characterisation using various analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR). However, there are two potential problems associated with this approach: (a) we and others have demonstrated that the oligosaccharides larger than disaccharides are mainly responsible for the anti-inflammatory effects and (b) depolymerisation may impart other process specific structural changes in the digested oligosaccharides [116, 117]. Hence, the research goal should be to isolate and characterise the structurally unmodified polysaccharides, in part to explore their anti-inflammatory activity. In considering these two problems it must be recalled that

currently available advanced analytical techniques such MS and NMR are not capable of characterising the mixtures of heterogeneous polysaccharides present in intact heparin. However, an ion-exchange chromatography has been recently developed to separate and isolate polysaccharides of heparin without their structural modification, and the continued efforts of structural chemists to improve the resolution power of new hyphenated techniques (e.g. liquid-chromatography-MS/MS and liquid-chromatography-NMR) will increase the efficiency of structural characterisation of ion-exchange derived oligosaccharides. However, structural characterisation and development of non-anticoagulant formulations of heparin will require extensive validation and formulation steps, and is likely to take a long time to eventuate. In the meantime, using murine experimental models we aim to focus on investigating the long-term anti-inflammatory effectiveness and safety of different doses of various sized oligosaccharides.

CHAPTER TWO

Opposing Effects of Low Molecular Weight Heparins on the Release of Inflammatory Cytokines from Peripheral Blood Mononuclear Cells of Asthmatics

2.1 ABSTRACT

***Background**:* T-cell-mediated inflammatory cytokines, such as interleukin (IL)-4, IL-5, IL-13 and tumor necrosis factor-alpha (TNF-α), play an important role in the initiation and progression of inflammatory airways diseases. Low-molecular-weight heparins (LMWHs), widely used anticoagulants, possess anti-inflammatory properties making them potential treatment options for inflammatory diseases, including asthma. In the current study, we investigated the modulating effects of two LMWHs (enoxaparin and dalteparin) on the release of cytokines from stimulated peripheral blood mononuclear cells (PBMCs) of asthmatic subjects to identify the specific components responsible for the effects.

Methods: PBMCs from asthmatic subjects (consist of ~75% of T-cells) were isolated from blood taken from ten asthmatic subjects. The PBMCs were pre-treated in the presence or absence of different concentrations of LMWHs, and were then stimulated by phytohaemagglutinin for the release of IL-4, IL-5, IL-13 and TNF-α. LMWHs were completely or selectively desulfated and their anticoagulant effect, as well as the ability to modulate cytokine release, was determined. LMWHs were chromatographically fractionated and each fraction was tested for molecular weight determination along with an assessment of anticoagulant potency and effect on cytokine release.

Results: Enoxaparin inhibited cytokine release by more than 48%, whereas dalteparin increased their release by more than 25%. The observed anti-inflammatory effects of

enoxaparin were independent of their anticoagulant activities. Smaller fractions, in particular dp4 (four saccharide units), were responsible for the inhibitory effect of enoxaparin. Whereas, the larger fractions, in particular dp22 (twenty two saccharide units), were associated with the stimulatory effect of dalteparin.

Conclusion: Enoxaparin and dalteparin demonstrated opposing effects on inflammatory markers. These observed effects could be due to the presence of structurally different components in the two LMWHs arising from different methods of depolymerisation. This study provides a platform for further studies investigating the usefulness of enoxaparin in various inflammatory diseases.

2.2 INTRODUCTION

Unfractionated heparin (UFH), a member of the glycosaminoglycan family, is a complex heterogeneous mixture of polysulfated chains comprised of alternating disaccharide residues of D-glucosamine and uronic acid residues linked by 1→4 glycosidic bonds [128]. The well-known biological role of UFH is its ability to influence blood coagulation and it has been extensively used in clinical practice as an anticoagulant [129]. In recent years, UFH has largely been replaced by low-molecular-weight heparins (LMWHs) for the treatment and prophylaxis of deep vein thrombosis because of more favourable pharmacokinetic properties and with a reduced rate of side effects [6, 130]. LMWHs are modified derivatives of UFH obtained by either chemical or enzymatic depolymerisation of UFH [131]. The key structural unit of heparins (UFH and LMWHs) responsible for their anticoagulant activity consists of three D-glucosamine and two uronic acid residues (known as a pentasaccharide sequence). This pentasaccharide sequence binds to the serine protease inhibitor anti-thrombin III and induces conformational changes within the structure of

anti-thrombin, thereby accelerating its interaction and subsequent inhibition of thrombin and/or factor Xa of the coagulation cascade [5]. However, not all fragments, also known as oligosaccharides, within heparins contain an anti-thrombin specific pentasaccharide sequence. For example, merely 20-50% of the oligosaccharides of UFH contain the specific anti-thrombin binding domain and the bulk of the oligosaccharides are composed of relatively non-specific sequences, also known as non-anticoagulant oligosaccharides [10, 132].

It is now recognised that besides the well-recognised anticoagulant effect, heparins also exhibit a broad spectrum of anti-inflammatory and immune-modulating properties [36, 41, 133-135]. The anti-inflammatory effect of heparins is thought to be due to their ability to alter the activity of a wide range of proteins, such as adhesion molecules, growth factors, cytotoxic mediators and tissue-destructive enzymes [13]. Clinical studies have reported the successful use of heparins for the treatment of chronic obstructive pulmonary disease [36], cancer [133], ulcerative colitis (UC) [134] and lichen planus [135]. The anti-angiogenesis effect in cancer has been shown to be mediated through suppression of tumor vascular endothelial growth factor expression [136]. Anti-UC properties are thought to be exhibited by inhibiting the recruitment of neutrophils, as well as healing of ulcerated mucosa by restoring the high-affinity receptor binding of fibroblast growth factor [137]. Similarly, the therapeutic effectiveness of heparins in lichen planus is thought to be mediated by competitive inhibition of an important component of the extracellular matrix, known as heparinase [109].

A number of clinical studies have also reported the beneficial effects of heparins in asthma [35, 40-42, 95]. However, the mechanisms behind these effects are

not well understood and there are plausible inflammatory pathways that remain to be explored. Despite being a complex disorder, the aetiology and pathophysiology of asthma is relatively well understood. Cytokines play a pivotal role in orchestrating the inflammation and structural changes of the airways in asthma. Among several types of important inflammatory mediators, T-cell mediated cytokines are known to be the key drivers of respiratory inflammation [138]. During early respiratory inflammation, the activated *naïve* T-helper cells release several inflammatory mediators, including IL-4, IL-5 and IL-13 [23]. IL-4 and IL-5 rapidly attract and prime eosinophils and mast cells. These cells, when activated, release high quantities of IL-5 and tumor necrosis factor (TNF)-α [139]. TNF-α sustains lung inflammatory responses by increasing the accumulation and activation of neutrophils and eosinophils in the airways. Their activation triggers the release of cytotoxic products, further damaging the airways [140]. IL-13 is associated with airway hyper-responsiveness, mucus production and structural changes in the airways called airway remodelling [141]. Also, IL-13 is known to play a key role in corticosteroid-resistant asthma by diminishing binding affinity between corticosteroids and their receptor ligands present on the surface of immune cells [142]. This is clinically significant as it is estimated that up to 10% of asthmatic patients have difficult-to-treat asthma that is often resistant to first line treatment with inhaled corticosteroids [143]. Given the high prevalence of asthma [144], 10% of asthmatic patients represent a significant number. Therefore, the development of potential therapeutic agents targeting difficult-to-treat asthma is highly desirable.

Because of the ability of highly negatively charged heparins to interact with a wide range of biological molecules, we and others have postulated that the possible anti-asthmatic activity of heparins is caused by their inhibitory effects on the release

of important inflammatory mediators involved in the pathogenesis of asthma [145-147]. Therefore, in the current *ex-vivo* study we investigated the ability of two widely used LMWHs (enoxaparin and dalteparin) to modulate the T-cell mediated release of IL-4, IL-5, IL-13 and TNF-α in asthmatic subjects, with the aim of identifying the specific oligosaccharide(s) responsible for the anti-inflammatory activity of the parent LMWH.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Enoxaparin (Clexane, 20 mg/0.2 mL; 2,000 IU/0.2 mL) was obtained from Aventis Pharma Ltd. (NSW, Australia). Dalteparin (Fragmin, 16 mg/0.2 mL; 2500 IU/0.2 mL) was purchased from Pfizer Inc. (NSW, Australia). Fondaparinux (Arixtra, 2.5 mg/0.5mL) was purchased from GlaxoSmithKline (Victoria, Australia). RPMI-1640 cell culture medium, Histopaque, antibiotics (penicillin G and streptomycin), phytohaemagglutinin (PHA), ethanol, hydrogen peroxide, sodium hydroxide, acetic acid, potassium hydroxide, sodium sulfate and lactate dehydrogenase (LDH) activity assay kits were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Fetal bovine serum was obtained from Invitrogen (Grand Island, NY, USA). ELISA kits for IL-4, IL-5, IL-13 and TNF-α were purchased from Mabtech Australia Pty. Ltd. (Victoria, Australia). Fluroaldehyde assay reagent was purchased from Pierce (Rockford, IL, USA). The anti-factor Xa (AFXa) assay kit was purchased from American Diagnostica (Stamford, CT, USA). Ultrafiltration disk membranes were purchased from Millipore (NSW, Australia). Heparin-derived unsaturated oligosaccharide standards were purchased from V-LABS (Covington, LA, USA).

2.3.2 Study sample

A total of 10 healthy (mean age: 34.4 years; range: 28-48 years, 8 males, 2 females) and 10 asthmatic (mean age: 45.6 years; range: 39-51 years, 4 males, 6 females) subjects were recruited from the Medical Science Precinct, University of Tasmania, Australia. The healthy volunteers were not suffering from any acute or chronic diseases and the asthmatic subjects were suffering from no other diseases apart from mild asthma. The asthmatic subjects had not consumed systemic or inhaled corticosteroids or any other immunomodulatory medications to control their asthma in the 2 months before the blood samples were drawn. No information of either the use of other medications or forced expiratory volume (FEV1) was obtained from the recruited participants.

2.3.3 Ethics statement

All volunteers were recruited by invitation and the research protocol was approved by the Health and Medical Human Research Ethics Committee (Tasmania, Australia) Network (Approval number: H0013117). Written informed consent for the collection of blood samples was obtained from the recruited volunteers.

2.3.4 Isolation of peripheral blood mononuclear cells (PBMCs)

Whole blood (80 mL) from each volunteer was collected and mixed with an equal volume of incomplete RPMI-1640 culture medium to reduce the density of blood, allowing efficient separation of PBMCs from other cells. Aliquots of blood (30 mL) were layered over 20 mL of Histopaque (a density gradient cell separation medium). Following centrifugation at 200g for 30 minutes at 20°C (Eppendorf; Model: 5810R), PBMCs were aspirated from the Histopaque/aqueous interface and centrifuged at

700g for 10 minutes. Cells were washed twice with serum-free medium and resuspended in complete medium [RPMI-1640 supplemented with 2.0 mM L-glutamine, 10% fetal bovine serum and antibiotics (penicillin G and streptomycin)].

2.3.5 Preparation of stock solutions

Stock solutions of enoxaparin, dalteparin and fondaparinux at 1 mg/mL were prepared in RPMI-1640 medium and filtered sterile through 0.2 µm pore size syringe filters (Pall Life Sciences, Victoria, Australia). Similarly, stock solution of PHA (2.5 mg/mL in RPMI-1640 medium; stored at -20°C) was prepared. The stock solutions of LMWHs were serially diluted with RPMI-1640 medium to obtain concentrations over the range of 5 to 1000 µg/mL. Likewise, PHA was diluted with RPMI-140 medium to obtain a concentration of 10 µg/mL.

2.3.6 Stimulation and LMWHs treatment of PBMCs

PBMCs were cultured in 24-well cell culture plates at a concentration of 1×10^6 cells/mL/well and stimulated with 10 µg/mL of PHA (T-cell specific stimulant) in the presence of either RPMI-1640 medium (negative control), different concentrations (5, 10, 20, 50, 80 and 100 µg/mL) of enoxaparin or dalteparin, or 5 to 1000 µg/mL of fondaparinux. After 72 hours of incubation (37°C, humidified 5% CO_2 atmosphere), PBMC cultures were centrifuged, and supernatants were removed and analysed for the levels of released cytokines (IL-4, IL-5, IL-13 and TNF-α) using enzyme-linked immunosorbent assay (ELISA).

2.3.7 ELISA

Each high protein binding 96-well ELISA plate was prepared as per manufacturer's recommendations. Briefly, plates were coated with 100 µL/well of capture antibody

(diluted in ELISA diluent) and incubated overnight at 4-8°C. The plates were washed twice with wash buffer (200 µL/well); blocked by addition of blocking buffer (PBS with 0.05% Tween 20 containing 0.1% bovine serum albumin) and incubated for 1 hour at room temperature before washing again for 5 times. Stock solutions of cytokine standards were prepared in ELISA diluent. PBMC supernatants and cytokine standards (100 µL/well) were added to plates, incubated for 2 hours and washed 5 times. Next, 100 µL/well of biotinylated detection antibody, at a concentration of 1 µg/mL, was added to each plate and washed 5 times after incubating for 1 hour. Further, 100 µL/well of standard horseradish peroxidase conjugated streptavidin (a commonly used enzyme to modify substrate resulting in colour development) was added to each plate and incubated for 1 hour. Plates were again washed for five times and 3,3',5,5'-tetramethylbenzidine as a chromogenic substrate solution (100 µL/well) was added. Plates were then allowed to stand in dark for 20-30 minutes and the reaction was quenched using 50 µL/well of stop solution (1N hydrochloric acid). Measurement of the optical density was performed using a plate reader (Spectra Max M2 microplate reader, Sunnyvale, CA) at 450 nm. Each PBMC treatments were performed in triplicates and supernatants of each treatment was analysed in duplicate.

2.3.8 PBMC viability and cytotoxicity assay

The effect of LMWHs on viability of cells after 72 hours of incubation was assessed using two methods. Firstly, a trypan blue exclusion test was performed on cells and their viability was determined by counting the unstained cells (cells which did not take up trypan blue) using a haemocytometer, as described previously [148]. Secondly, the activity of LDH in culture supernatant was tested to investigate the cytotoxic effect of LMWHs using a LDH in-vitro toxicology assay, as described

before [149]. The LDH assay kit was prepared as per the manufacturer's instructions. Briefly, PBMC culture supernatants were centrifuged at 250g for 4 minutes. An aliquot containing 50 µL of either blank (complete medium), control (PBMCs only), cells treated with PHA alone or with PHA in the presence of 100 µg/mL enoxaparin or dalteparin was mixed with 100 µL of solution containing LDH assay mixture (LDH substrate, LDH dye and LDH cofactor). The mixture was then covered with aluminium foil and incubated at room temperature for 20-30 minutes for colour development and the reaction was terminated by the addition of 1 N hydrochloric acid (15 µL). The absorbance was measured spectrophotometrically using a plate reader (Spectra Max M2 microplate reader, Sunnyvale, CA) at a wavelength of 490 nm. Each sample was prepared and analysed in triplicate. Lastly, cell proliferation was carried out by counting the total number of cells 72 hours after PBMC treatments.

2.3.9 Desulfation of LMWHs

2.3.9.1 *Complete desulfation of LMWHs*

A solution containing 5 mg/100 µL of enoxaparin and dalteparin was subjected to acid hydrolysis for complete removal of sulfate groups, as described previously [150]. Briefly, 1.5 mL of nitric acid was added to each sample in a capped glass vial and the solution was heated at 80°C overnight before adding 0.3 mL of hydrogen peroxide. The temperature was further raised to 110°C for the following 6 hours. An experimentally determined volume of 1 M sodium hydroxide was used to neutralise the mixture containing nitric acid, hydrogen peroxide and enoxaparin or dalteparin. The neutralised solution was diluted with 8 mL of Milli-Q water and 400 µL of this solution was further diluted to 8 mL.

2.3.9.2 *N-desulfation of LMWHs*

A solution containing 5 mg/100 µL of enoxaparin or dalteparin was incubated at 50°C for 30 minutes in the presence of tetrahydrofuran (650 µL) and Milli-Q water (50 µL) for partial *N*-desulfation, as described previously [151] with minor modifications. The mixture was neutralised using 0.1 M sodium hydroxide. The resulting product was evaporated to dryness and precipitated by the addition of anhydrous methanol (80% *v/v*) followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was carefully discarded and samples were kept at 4°C overnight. Any remaining traces of methanol were removed using a miVac DNA centrifugal concentrator (Genevac Ltd, Suffolk, UK) and the precipitants were dissolved in 5 mL Milli-Q water.

2.3.9.3 *Selective 2-O and 3-O-desulfation of LMWHs*

Selective 2-*O* and 3-*O*-desulfation was performed using a previously described method [151]. Briefly, a solution containing 5 mg/100 µL of enoxaparin or dalteparin was dissolved in 0.1 M sodium hydroxide (200 µL). The solution was then frozen and lyophilised to dryness. The residues were dissolved in Milli-Q water (0.5 mL) and the pH was adjusted to 8 by the addition of acetic acid solution. The solution was dialysed against water for 2 days and lyophilised to obtain 2-*O*, 3-*O*-desulfated enoxaparin and dalteparin fragments.

Free sulfate content, free amino groups and anticoagulant activity of completely and selectively desulfated enoxaparin samples was investigated, along with their effects on the PHA-induced release of cytokines from activated PBMCs of the asthmatic subjects.

2.3.10 Ion chromatography analysis of free sulfate content

Free sulfate content of completely and selectively desulfated LMWHs was determined using a previously developed ion chromatography (IC) method [116]. Samples were injected into a Dionex DX-120 instrument (Sunnyvale, CA, USA) consisting of a GP50 gradient pump and AS50 auto sampler. Mobile phases were composed of potassium hydroxide (KOH) and Milli-Q water. Hydroxide eluent gradients were prepared through mixing of KOH solution and Milli-Q water online using a Dionex EluGen II KOH cartridge. A Dionex IonPac AS11 column was used to detect the sulfate content with the optimised KOH eluent gradient from 1 mM to 15 mM over a period of 15 minutes. A total flow rate of 1.0 mL/minute and an injection volume of 25 µL was maintained. Conductivity detection in suppression mode was carried out using a CD25 conductivity detector. Instrument control and data acquisition were performed using Chromeleon® software. Sodium sulfate containing 0-20 µg/mL of sulfate was used to prepare the standard curves. For comparison, the presence of free sulfate content in intact LMWHs (before desulfation) was also investigated after ultra-filtration of enoxaparin/dalteparin at 15000 rpm for 10 minutes. Each sample was prepared in triplicate and analysed in duplicate.

2.3.11 Analysis of free amino groups

The determination of free amino groups in selectively desulfated LMWHs was determined using a sensitive fluoraldehyde-based assay, as described previously [117]. Briefly, selectively desulfated samples (20 µL) were mixed with deionised water (180 µL) before the addition of fluoraldehyde assay reagent (1 mL). The solution was mixed well and fluorescence was measured at 455 nm after excitation at

360 nm using a fluorescence spectrophotometer (model 1605-10S, Perkin-Elmer, Tokyo, Japan). Standard curves (0 to 600 µg/mL) were prepared using glycine.

2.3.12 Analysis of anticoagulant activity

The potentiating effect of intact or desulfated LMWHs on anti-thrombin III inhibition of activated factor Xa was determined as previously described [116]. Briefly, a low-volume microtitre plate anti-FXa assay was performed by incubating (3 minutes, 37°C) a solution containing anti-thrombin III, FXa and intact or desulfated LMWH samples, followed by addition of FXa substrate and further incubation for 10 minutes. The reaction was quenched using glacial acetic acid and the intensity of developed colour was spectrophotometrically measured at 405 nm (Multiskan Go, SkanIt software, Thermo Fisher Scientific).

2.3.13 Fractionation and collection of LMWH fractions

Fractionation of enoxaparin and dalteparin was performed using a previously developed high-performance size-exclusion chromatography (HP-SEC) method [116] with some modifications. A high-performance liquid chromatography (HPLC) system consisting of a Prostar 230 solvent delivery module, a Prostar 335 DAD detector and a Prostar 410 autosampler (Varian, Melbourne, Australia) was utilised. Data acquisition and instrument control were carried out using Star Chromatography Workstation® software. The analyses were performed by injecting 10 mg/mL of enoxaparin or dalteparin using a 200 µL sample loop and a 70 µL sample injection volume. UV detection was monitored at 232 nm. Isocratic elution of LMWHs was performed on a Superdex™ 10/300 GL (300×10 mm) size-exclusion column (GE Healthcare Bio-Sciences, Uppsala, Sweden) using 0.3 M sodium chloride eluent at a flow rate of 0.2 mL/minutes. After each HP-SEC injection (n=20), 8 fractions of

enoxaparin and 9 fractions of dalteparin were collected. The collected fractions were concentrated on a miVac DNA centrifugal concentrator at 40°C and subsequently desalted using PD MidiTrap G-10 columns (GE Healthcare Life Sciences, Uppsala, Sweden). Each fraction was filter sterilized using 0.2 µm pore size syringe filters (Pall Life Sciences, Victoria, Australia) and stored at 4°C until further use. Fractions were analysed in triplicate for their effects on PHA-induced release of cytokines, as well as for their anticoagulant activity as described above.

2.3.14 Statistical analysis

Data are presented as mean ± standard deviation (SD) or as percentage change in the release of cytokines following treatments (enoxaparin, dalteparin, desulfated enoxaparin/dalteparin or HPSEC-derived fractions of enoxaparin/dalteparin) compared to the control samples. Statistical analysis was performed using GraphPad Prism (version 6, GraphPad Software Inc, CA, USA), and significance was evaluated using independent sample or paired Student's *t*-test, and one way analysis of variance (ANOVA), where applicable, followed by Dunnett's multiple comparison test. A *p*-value of <0.05 was considered statistically significant.

2.4 RESULTS AND DISCUSSION

2.4.1 Release of cytokines from stimulated PBMCs

The levels of the four cytokines (pg/mL) measured 72 hours after PHA-induced stimulation of PBMCs from healthy and asthmatic volunteers are shown in Figure 2.1. The calibration curves used for the measurement of cytokines were generated using seven recommended concentrations of respective cytokine standards. The linearity, estimated by correlation coefficient (r^2), was greater than 0.964 for each of the

cytokines. As expected, the levels of tested cytokines from PBMCs of asthmatic subjects were significantly higher than those released from healthy volunteers. It is known that the relative concentrations of the two main types of T-helper cells (Th1 and Th2) are different in patients with asthma compared to healthy individuals. In the healthy population, Th1/Th2 balance is maintained by producing the required Th1 or Th2 cells to establish the normal immune tolerance [152]. In patients suffering from allergic disorders, including asthma, the Th1/Th2 balance becomes abnormal and it shifts abruptly towards Th2 cells [152]. The greater population of Th2 cells upon activation release various cytokines, including IL-4, IL-5, IL-13 and TNF-α.

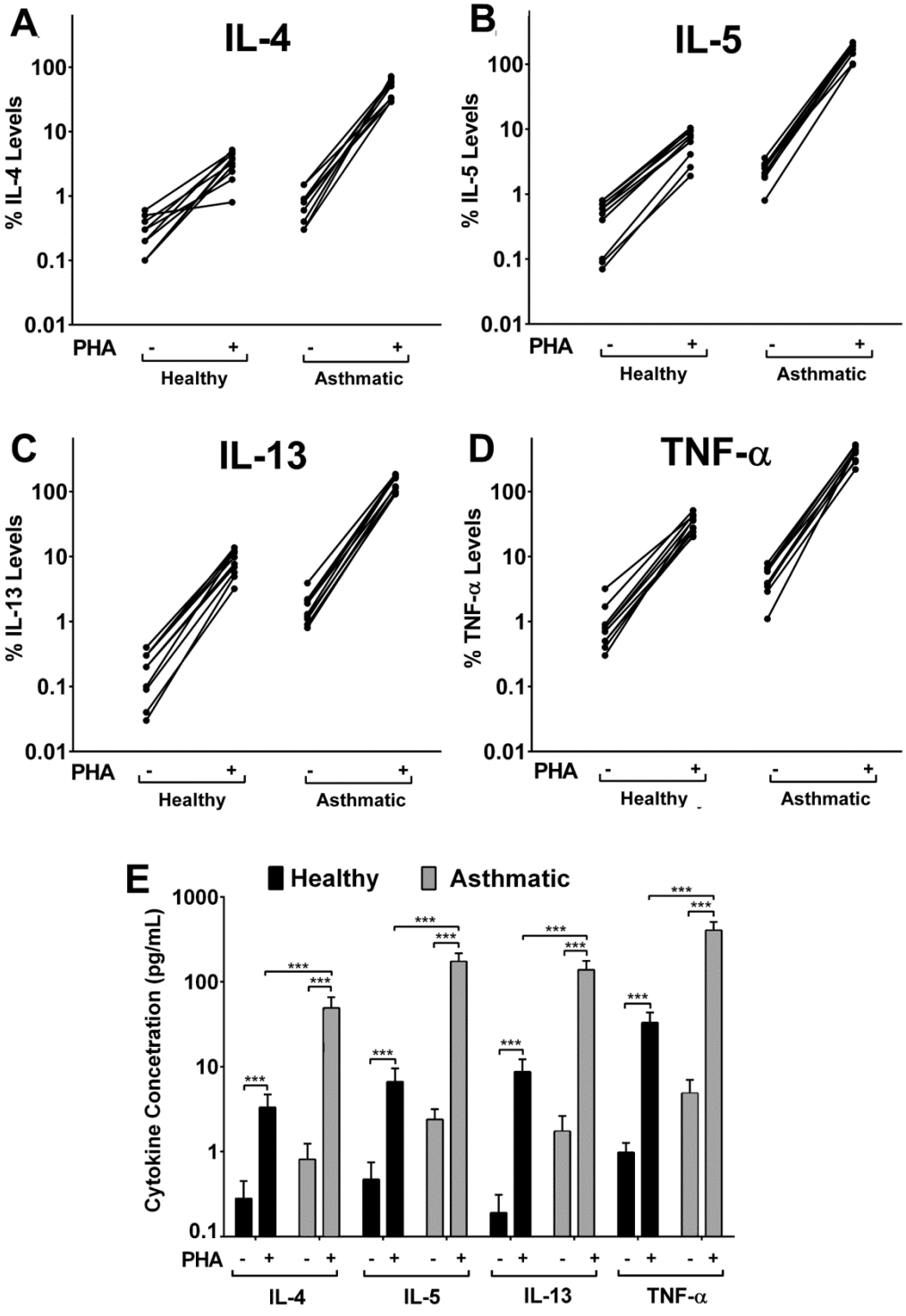

Figure 2.1 *Ex-vivo* cytokine release. PHA-induced release of IL-4, IL-5, IL-13 and TNF-α in PBMC culture supernatants of healthy (n=10) and mild asthmatic (n=10) subjects **(A to D)**. Error bars are omitted for reasons of clarity. **(E)** Summary of the results from A to D. Each sample was analysed in triplicate. Error bars indicate the standard deviation. ****p*<0.001 versus PHA-stimulated control.

2.4.2 Effect of LMWHs on cytokine release

To investigate the effects of LMWHs on the release of cytokines, enoxaparin or dalteparin was added to the cells prior to the addition of PHA. The percentage inhibition of cytokine release in the presence or absence of enoxaparin and dalteparin is shown in Figure 2.2. The inhibitory effect of enoxaparin was found to be concentration-dependent and its maximum effect was observed at 50 μg/mL (Figure 2.2A to 2.2D). The release of IL-4, IL-5, IL-13 and TNF-α was inhibited by more than 58%, 50%, 55% and 48%, respectively, in the presence of 50 μg/mL of enoxaparin. On the other hand, dalteparin failed to supress the release of tested cytokines and, on the contrary, the release of IL-4, IL-5, IL-13 and TNF-α was increased by more than 25% in the presence of 80 or 100 μg/mL of dalteparin (Figure 2.2A to 2.2D).

Enoxaparin and dalteparin are prepared by different depolymerisation processes [131]. Enoxaparin (an average molecular weight of 4500Da) is prepared by chemical β-eliminative cleavage of benzyl ester of UFH and dalteparin (an average molecular weight 6000Da) is prepared by nitrous acid induced deaminative cleavage of UFH. Therefore, their oligosaccharides have different sequences as well as terminal reducing and non-reducing ends. Compared to dalteparin, enoxaparin is more heterogeneous in nature and is mainly composed of oligosaccharides ranging from dp2 (two saccharide units) to dp16 (16 saccharide units) [153]. On the other hand, the smallest oligosaccharide unit found in dalteparin is dp8 and it mainly contains oligosaccharides longer than dp12 [153].

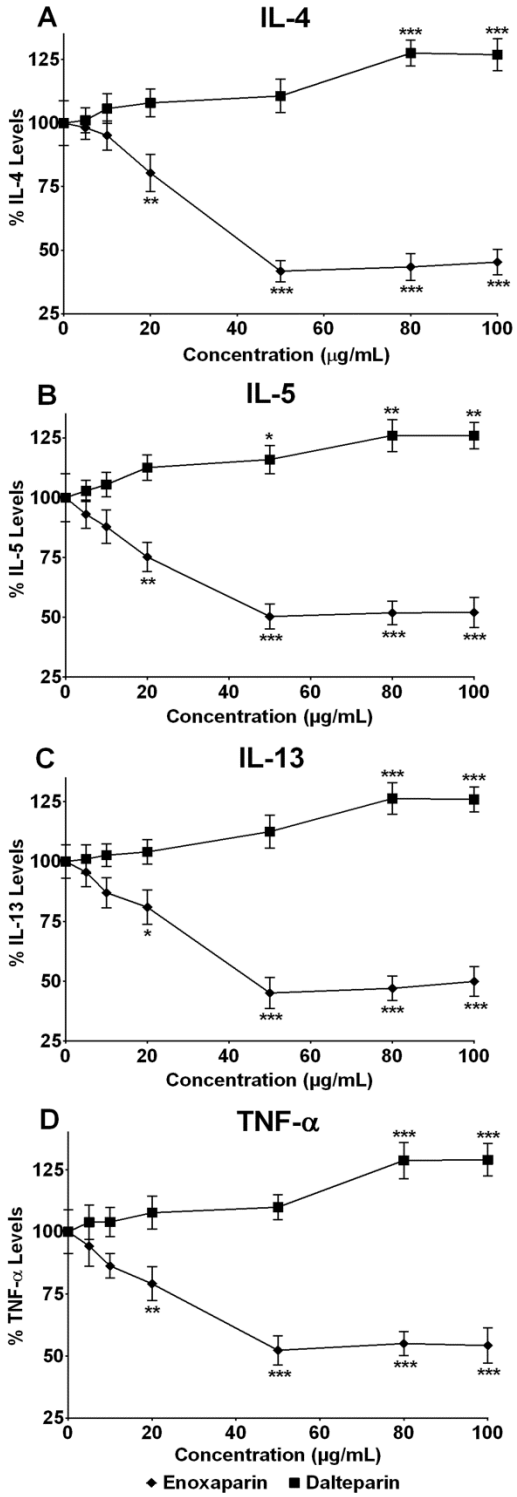

Figure 2.2 Concentration-dependent effect of LMWHs on cytokine release. Effect of different concentrations of enoxaparin and dalteparin on cytokine levels [IL-4 **(A)**, IL-5 **(B)**, IL-13 **(C)** and TNF- α **(D)**] following PHA-induced *ex-vivo* stimulation of PBMCs from asthmatic subjects (n=10). Data is presented as percentage (mean ± SD) of the maximal observed cytokine concentrations. **p*<0.05, ***p*<0.01 and ****p*<0.001 versus PHA-stimulated control.

In line with our results, other studies have also shown different responses of various cells and molecules involved in the process of inflammation to LMWHs. It has been proposed that different effects might result from the presence of different proportions of small and large oligosaccharide chains within various LMWHs [154, 155]. Based on this, it can be postulated that the suppression of cytokine release could be due to shorter oligosaccharide chains (≤dp8), prevalent in enoxaparin, whereas an increase in cytokine release might be due to longer oligosaccharides (>dp8), which are common in dalteparin. It has been shown that Th1-type cytokines such as interferon-γ and IL-12 play an important role in controlling immune and allergic responses in asthma [156]. For example, interferon-γ may counteract Th2 mediated immune responses by 1) minimising the development of Th2 cells, 2) increasing the production of IL-12, 3) inducing apoptosis of eosinophil and 4) preventing immunoglobulin switch in B cells. The current study investigated the effects of LMWHs on the release of Th2- but not Th1-type cytokines. Therefore, future studies should be focused on determining the effects of LMWHs on the level of Th1-type cytokines as well.

2.4.3 Effect of LMWHs on viability and proliferation of PBMCs

Different types of heparins have shown to possess pro- or anti-proliferative effects [157, 158]. Therefore, it was important to determine whether or not the observed effect above on cytokine production was due to either enoxaparin-induced cell toxicity or dalteparin-induced cell proliferation. The cell viability was assessed in the presence of PHA alone or in combination with PHA and enoxaparin/dalteparin, by detecting the release of LDH (Figure 2.3A). Neither enoxaparin nor dalteparin increased the release of LDH, whereas PHA, as described before, induced cellular toxicity [159, 160]. PHA-induced toxicity was not affected when PHA-stimulated

cells were co-incubated with enoxaparin or dalteparin. Cellular viability in the presence or absence of enoxaparin and dalteparin is shown in Figure 2.3B. Unlike PHA, enoxaparin or dalteparin did not produce signs of cellular cytotoxicity. Cell proliferation in the presence or absence of enoxaparin and dalteparin is shown in Figure 2.3C. The proliferation of PBMCs was found to be significantly increased in the presence of PHA, whereas enoxaparin and dalteparin showed no effect on cell proliferation. These results indicate that modulation of cytokine release in the presence of tested LMWHs was not related to their cytotoxicity or changes in cellular proliferation.

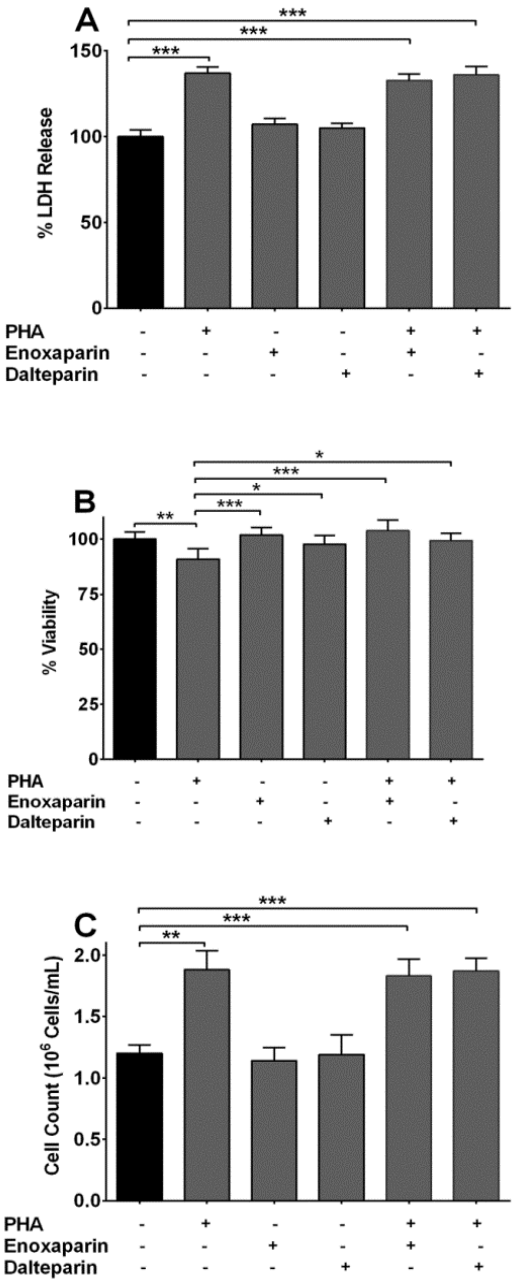

Figure 2.3 Effect of LMWHs on cellular viability. (A) LDH activity of PBMC supernatants from asthmatic subjects (n=5) expressed as the percentage of maximum LDH release following 72 hours of incubation in the presence of either PHA, enoxaparin/dalteparin alone or enoxaparin/dalteparin co-incubated with PHA. Error bars indicate standard deviation. ***p<0.001 compared to unstimulated cells only control. **(B)** Effect of enoxaparin and dalteparin on the viability of PBMCs obtained from asthmatic subjects (n=5) after pre-treatment of cells with either PHA, enoxaparin/dalteparin alone or enoxaparin/dalteparin co-incubated with PHA. Viability was measured using trypan blue dye exclusion test 72 hours after incubation with drugs and is presented as the percentage of viable cells. Error bars indicate standard deviation. *p<0.05, **p<0.01 and ***p<0.001 compared to either unstimulated cells only or PHA-stimulated control. **(C)** The effects of enoxaparin and dalteparin on PHA-induced proliferation of PBMCs obtained from asthmatic subjects (n=5) measured 72 hours after incubation with enoxaparin/dalteparin alone or enoxaparin/dalteparin co-incubated with PHA. Error bars indicate standard deviation. **p<0.01 and ***p<0.001 compared to either unstimulated cells only or PHA-stimulated control.

2.4.4 Effect of desulfated LMWHs on cytokine release

The anticoagulant activity of LMWHs is strongly influenced by the degree of sulfation and the distribution of sulfate groups in their oligosaccharide chains [128]. To investigate whether the observed effect of enoxaparin and dalteparin was dependent on their anticoagulant activity, they were subjected to complete or selective desulfation. Free sulfate in intact enoxaparin and dalteparin was determined by IC and was found to be equivalent to 0.42% and 0.36% (w/w), respectively. Total bonded sulfate in enoxaparin and dalteparin after complete desulfation, and allowing for the free sulfate, was found to be 38.7 and 42.3% (w/w), respectively. This value for enoxaparin or dalteparin was similar to the theoretical estimation of sulfate content (40% of enoxaparin and 44% w/w of dalteparin) based on 75% trisulfation of the heparin disaccharide repeating unit from porcine mucosa and an average of one *N*-acetyl group per parent heparin molecule and the type of their reducing ends (2-*N*, 6-*O*-disulfo-D-glucosamine or 1,6-anhydro groups for enoxaparin and 6-*O*-sulpho-2,5-anhydro-D-mannitol for dalteparin).

The effect of completely desulfated enoxaparin and dalteparin on the release of TNF-α (levels of which were found to be highest in PBMC supernatants) was examined. Unlike intact LMWHs, completely desulfated molecules did not inhibit or increase the release of cytokines, indicating the importance of sulfate groups for retaining the observed activity of LMWHs. The key structural unit of heparins that confers anticoagulant activity is an oligosaccharide sequence consisting of three D-glucosamine and two uronic acid residues. The anticoagulant activity of heparins has been shown to be dependent on *N*- and *O*-sulfate groups present in the oligosaccharide sequence. Elimination of the *N*-sulfate groups results in decreased anticoagulant

activity, but the elimination of 3-*O*-sulfate group of the central D-glucosamine results in the loss of anticoagulant activity by approximately 20,000 times [128].

To determine the role of *N*- and 2-*O*/3-*O* sulfate groups in any pro- or anti-inflammatory effect, enoxaparin and dalteparin were selectively desulfated. *N*-desulfation and 2-*O*/3-*O* desulfation was confirmed by commonly used fluoraldehyde assay and IC, respectively. It was found that enoxaparin and dalteparin retained their activity in the absence of *N*- and 2-*O*/3-*O* sulfate groups (Figure 2.4A and 2.4B) indicating these groups are not important for their observed effects. Therefore, the anti- or pro-inflammatory effects of LMWHs were independent of their anticoagulant activity.

Clinical studies have indicated that LMWHs can potentially be used in human inflammatory disorders [12, 147]. However, they possess both anticoagulant and non-anticoagulant oligosaccharides. Therefore, their potential use is hampered by the risk of bleeding in conditions other than where anticoagulation is required [161]. To investigate whether the anti-inflammatory effect of enoxaparin is separable from its anticoagulant effect, enoxaparin was separated and the oligosaccharide(s) responsible for its anti-inflammatory effect was identified before determining the anticoagulant effect of the identified oligosaccharide(s). Dalteparin was also separated to identify the oligosaccharide(s) responsible for its pro-inflammatory effect.

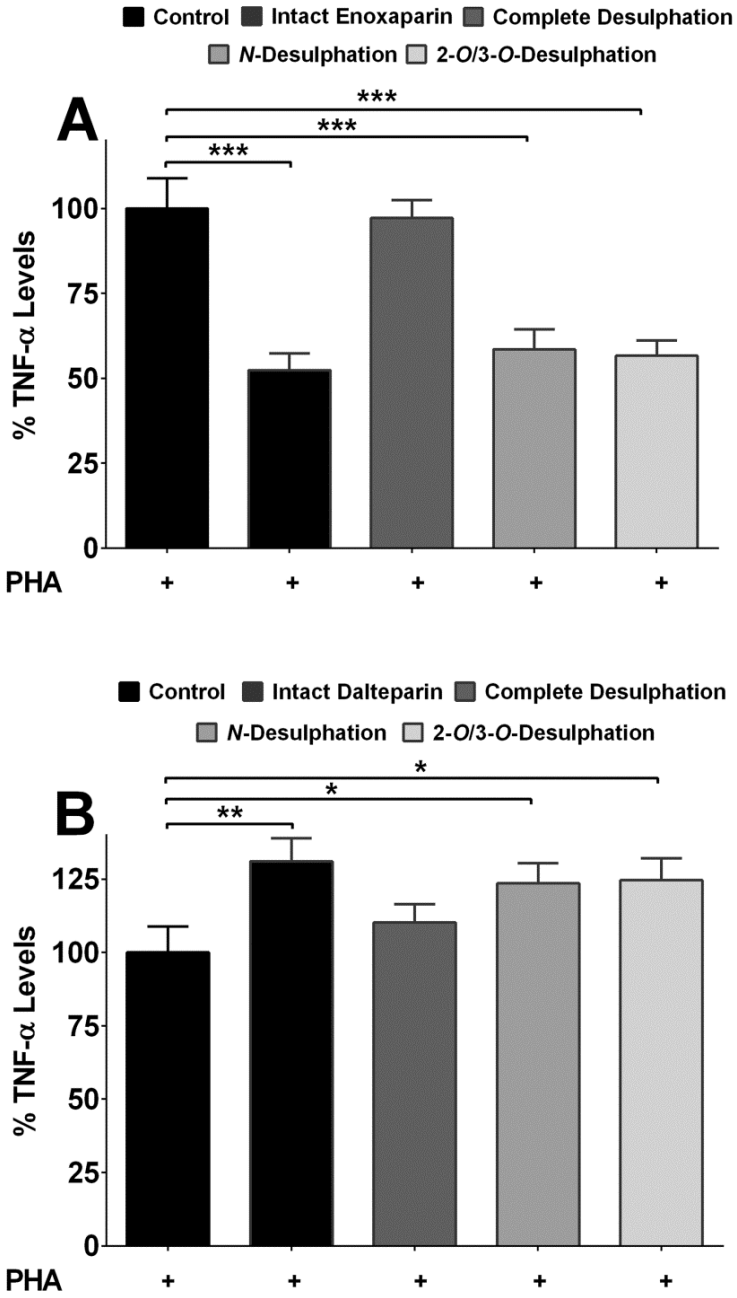

Figure 2.4 Effect of de-sulfated LMWHs on cytokine release. Suppression of TNF-α release by completely desulfated, *N*-desulfated or 2-*O*/3-*O*-desulfated fragments of enoxaparin **(A)** or dalteparin **(B)** after PHA-induced stimulation of PBMCs from asthmatic subjects (n=5). Error bars indicate standard deviation. **p*<0.05, ***p*<0.01 and ****p*<0.001 compared to PHA-stimulated control.

2.4.5 Separation and identification of oligosaccharides

The HP-SEC chromatograms of enoxaparin and dalteparin are presented in Figure 2.5. As expected, different chromatographic profiles of enoxaparin and dalteparin were observed. The saccharide composition of each HP-SEC separated peak of enoxaparin and dalteparin has been reported before [153]. Enoxaparin was separated into 8 different peaks representing dp2 to dp16, whereas dalteparin was separated into 9 different peaks representing dp8 to dp24 (Figure 2.5).

Due to structural complexity and high negative charge, LMWHs cannot be effectively separated without prior depolymerisation. Therefore, different techniques, such as reversed-phase high-performance liquid chromatography [162] and capillary electrophoresis [113], have been used for the separation of depolymerised heparin derivatives. However, elevated temperature or freeze drying processes used during depolymerisation can result in structural modifications of the oligosaccharides and therefore certain biological effects of intact LMWHs are lost or altered after depolymerisation process [116, 117]. Hence, enoxaparin and dalteparin in this study were separated using the HP-SEC method. This technique can separate oligosaccharides without the need for chemical or enzymatic depolymerisation of the parent molecule. Although this technique is most widely used for the identification of saccharide composition of LMWHs, it has its own specific limitations. For example, structurally different oligosaccharides having the same or similar saccharide composition cannot be separated using this technique. Therefore, it is possible that the HP-SEC separated fractions of LMWHs (e.g. dp4 of enoxaparin) are composed of structurally different molecules having the similar saccharide composition.

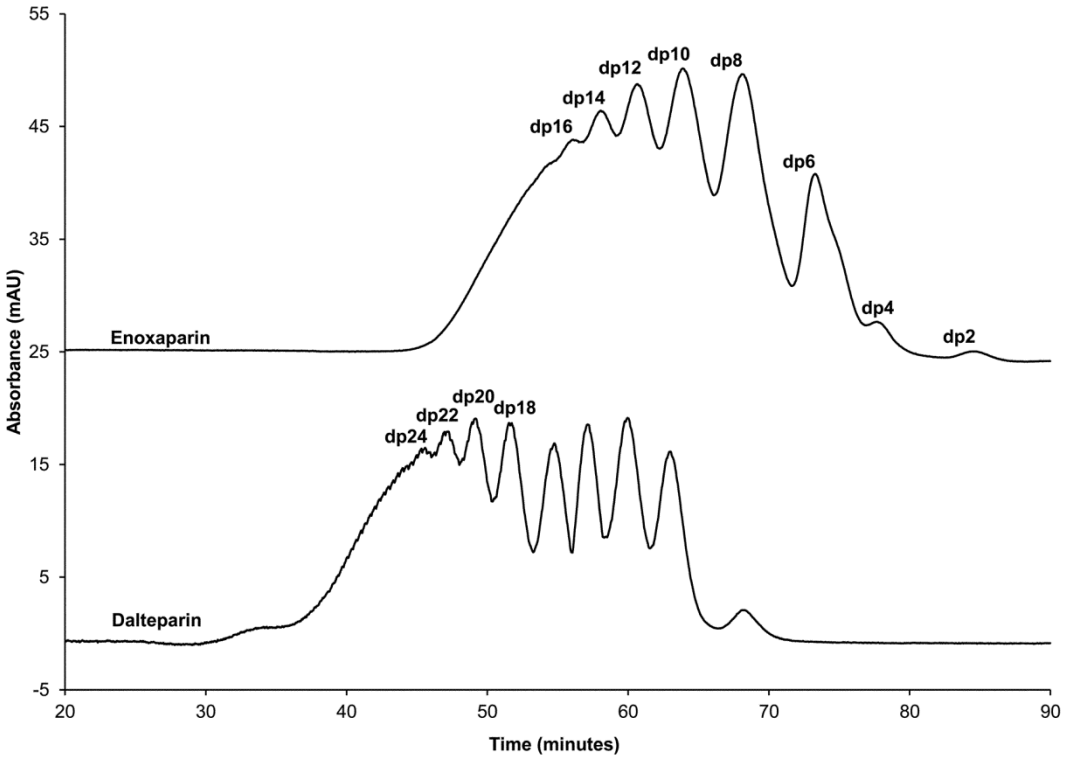

Figure 2.5 HPSEC analysis of enoxaparin and dalteparin. The HP-SEC methodology is described in the experimental section. Approximate saccharide composition of the separated peaks of enoxaparin and dalteparin is shown; dp2- two saccharide units to dp24- twenty four saccharide units.

2.4.6 Effect of identified oligosaccharides on cytokine release

Separated fractions of enoxaparin and dalteparin were collected and then re-analysed by HP-SEC to confirm their saccharide composition and purified using desalted columns. The ability of each desalted fraction to modulate the release of TNF-α is shown in Figure 2.6. The tested concentration of each fraction was based on its actual concentration, calculated using the peak area, present in 50 µg/mL of enoxaparin or 80 µg/mL of dalteparin. The release of TNF-α was significantly increased by 15%, 29% and 21% in the presence of dp20, dp22 and dp24 saccharides of dalteparin, respectively. On the other hand, dp8 to dp18 did not significantly modulate cytokine release, indicating the oligosaccharides larger than dp18 of dalteparin have pro-

inflammatory activity (Figure 2.6B). Fondaparinux, a synthetic LMWH, was used to determine whether the observed pro-inflammatory effect of dalteparin fractions were independent to their anticoagulant effects. Fondaparinux is composed of only pentasaccharide sequence responsible for the anticoagulant activity of LMWHs. It, at tested concentrations (5 to 1000 μg/mL), failed to inhibit or enhance the levels of IL-4, IL-5, IL-13 and TNF-α suggesting the observed effect of dalteparin molecules were not dependent to their anticoagulant activity.

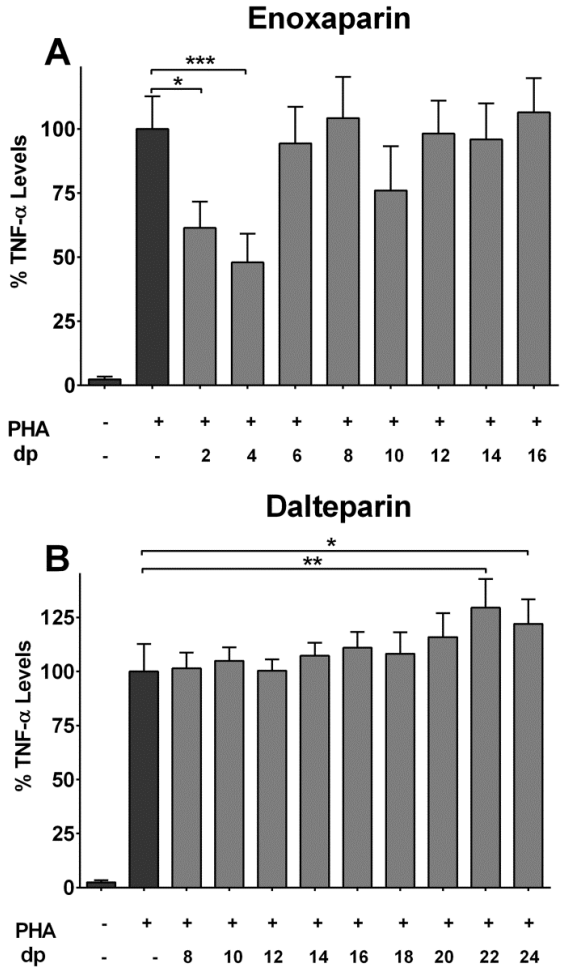

Figure 2.6 Effect of HPSEC fractions of LMWHs on cytokine release. Suppression of TNF-α release by eight HP-SEC derived saccharides (dp2 to dp16) of enoxaparin **(A)** or nine HP-SEC derived saccharides (dp8 to dp24) of dalteparin **(B)** after PHA-induced stimulation of PBMCs from asthmatic subjects (n=5). Error bars indicate standard deviation. *p<0.05, **p<0.01 and ***p<0.001 versus PHA-stimulated control.

Several autoimmune diseases are associated with down regulation of T-cell receptor signalling pathways, resulting in dysfunction of T-cells [163]. This may have important consequences such as insufficient response to various types of infections [164]. The therapeutic potential of dalteparin in such medical conditions could potentially be explored owing to its stimulatory effect on T-cells. HP-SEC separated dp2 and dp4 of enoxaparin inhibited the release of TNF-α by 39 and 52%, respectively (Figure 2.6A). Dp6 showed some activity but it was not statistically different from the control samples and the fractions larger than dp6 did not show a significant inhibitory effect (Figure 2.6A) suggesting the shorter oligosaccharides are responsible for the anti-inflammatory effect of enoxaparin. This finding is important because a minimum of five saccharide chain length with specific sulfation pattern is required for the anticoagulant activity of any type of LMWH. One of the major concerns against the development of LMWHs as an anti-asthmatic agent is the bleeding risk associated with its use. Disaccharide (dp2) or tetrasaccharide (dp4) chains are not sufficiently long enough to bind AT-III and therefore do not exhibit anticoagulant activity (which was confirmed by previously described low-volume microtitre plate anticoagulant assays). Hence, these fractions of enoxaparin would potentially minimise the risk of bleeding associated with intact enoxaparin and could be investigated further as potential therapeutic targets for the treatment of inflammatory diseases including asthma.

2.5 CONCLUSION

In conclusion, the current study provides a solid platform for further experimental and clinical studies. Future research should be designed focusing on: i) further separation of dalteparin's dp22 and enoxaparin's dp4 molecules, with the aim of identification

and structural elucidation of the saccharide moieties responsible for the pro- or anti-inflammatory activity of the parent LMWHs; ii) identification of underlying cellular and molecular mechanisms by which dp22 of dalteparin and dp4 of enoxaparin modulate the T-cell induced release of pro-inflammatory cytokines; and iii) pre-clinical and clinical studies confirming the observed non-anticoagulant activities of the identified enoxaparin oligosaccharides.

CHAPTER THREE

Ion-exchange Chromatographic Separation and Isolation of Oligosaccharides of Intact Low Molecular Weight Heparin for the Determination of their Anticoagulant and Anti-inflammatory Properties

3.1 ABSTRACT

Background: It is well-known that enoxaparin, a widely used anticoagulant and low-molecular-weight heparin containing a large number of oligosaccharides, possesses anti-inflammatory activity. While enoxaparin has shown promising results in various inflammatory disorders, some of its oligosaccharides have anti-inflammatory properties and others increase the risk of bleeding due to their anticoagulant effects. The aim of this study was to develop an effective ion-exchange chromatographic (IC) technique which allows the separation, isolation and consequently the identification of different oligosaccharides of enoxaparin with or without anticoagulant activity.

Methods: The developed method utilises a semi-preparative CarboPac PA100 (9 × 250 mm) ion-exchange column with sodium chloride gradient elution and UV detection at 232 nm. The anti-inflammatory activity of selected oligosaccharides was investigated using the Griess assay.

Results: The method successfully resolved enoxaparin into more than 30 different peaks. IC-derived oligosaccharides with high, moderate, low or no anticoagulant activity were identified using an anti-factor Xa assay. Using this technique, the oligosaccharides of enoxaparin with low or no anticoagulant activity, whilst exhibiting significant anti-inflammatory activity, could be fractionated.

Conclusion: This technique can provide a platform to identify the oligosaccharides which are devoid of significant anticoagulant activity and are responsible for the

therapeutic effects of enoxaparin that have been observed in various inflammatory conditions.

3.2 INTRODUCTION

Heparin is a naturally occurring endogenous substance, sequestered within the mast cells and found in granulated cells of organs and tissues, including the intestine and lung [165]. In clinical practice, heparin has been used as an anticoagulant for more than 50 years [129]. It is a heterogeneous mixture of highly sulfated and structurally unknown oligosaccharides belonging to the family of glycosaminoglycans [128]. As a therapeutic agent, heparin possesses both pharmacokinetic and pharmacological limitations, including variable bioavailability with subcutaneous administration, the propensity to interact with a wide range of plasma proteins, and the risk of inducing thrombocytopaenia and osteoporosis [6]. Therefore, in recent years heparin has been largely replaced by low-molecular-weight heparins (LMWHs), because of their improved pharmacokinetic properties, safety and efficacy [6, 130].

LMWHs are obtained by depolymerisation of unfractionated heparin and are composed of both anticoagulant and non-anticoagulant oligosaccharides [7]. LMWHs (e.g. enoxaparin) are primarily approved for the prophylaxis and treatment of deep-vein thrombosis [8]. However, it is now recognised that LMWHs exhibit a broad spectrum of anti-inflammatory and immune-modulating properties beyond their well-known anticoagulant effect [5, 58, 109, 166]. The anticoagulant activity of a LMWH is mainly dependent on the interaction between a specific pentasaccharide sequence constituting the antithrombin binding domain (Figure 3.1) and the serine protease inhibitor antithrombin-III (AT III) [5]. This sequence consists of three D-glucosamine and two uronic acid residues. Sulfate groups on the D-glucosamines are found to be

critical for retaining high anticoagulant activity. Elimination of any one of them results in a dramatic reduction in the anticoagulant activity. Removal of the unique 3-*O*-sulfate group results in complete loss of the anticoagulant activity [167, 168].

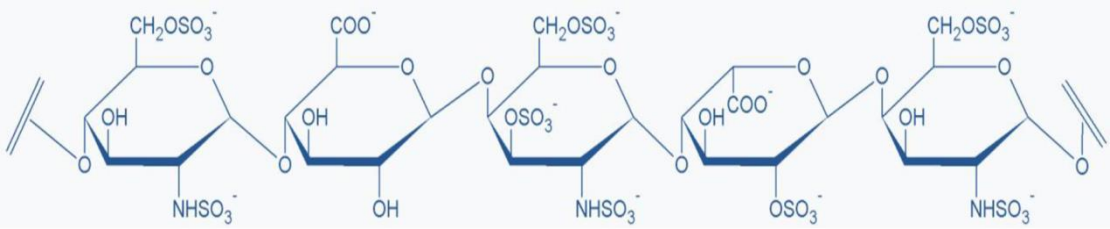

Figure 3.1 The unique pentasaccharide sequence of heparin having three D-glucosamine and two uronic acid residues. The anticoagulant activity of heparin is because of the binding between this sequence and AT III.

Interestingly, the potential therapeutic activities of LMWHs in a wide array of clinical disorders, such as asthma [58], ulcerative colitis [166] and lichen planus [109], depend largely on the interactions between its non-anticoagulant oligosaccharides and various biological molecules present in the extracellular fluid, extracellular matrix, intracellular granules or on the cell surface [13]. For instance, it has been reported that the non-anticoagulant oligosaccharides of heparin strongly bind and inhibit the functions of L- and P-selectins. These adhesive molecules are involved in the recruitment of leukocytes, a necessary first step in the inflammation cascade that occurs before the recruitment of various inflammatory mediators, including cytokines and integrins [33, 169]. However, the clinical use of a LMWH as an anti-inflammatory agent is largely limited in the presence of anticoagulant oligosaccharides, being associated with a significant risk of bleeding. To minimise such complications, low doses of LMWHs have been used, but the risk of bleeding still remains a major concern [109, 166, 170]. Also, the anti-inflammatory effects of

LMWHs are dose-dependent and at lower doses their anti-inflammatory response is reduced [171, 172].

Therefore, a clear rationale exists to separate and identify the non-anticoagulant oligosaccharides within the parent LMWH. This approach would provide an opportunity to separate the anticoagulant and anti-inflammatory activities of a LMWH. The current approach to identify the non-anticoagulant oligosaccharides of a LMWH is to perform enzymatic or chemical depolymerisation of the parent compound [33, 173]. By contrast, a chemical or enzymatic depolymerisation results in the structural modification of oligosaccharides and it has been demonstrated that certain biological aspects of glycosaminoglycans could indeed be removed by the depolymerisation process [115, 174]. Some oligosaccharides of LMWH are heat-sensitive and can undergo chemical modification, especially desulfation during the elevated temperatures of the depolymerisation process [116]. An oligosaccharide's sulfation pattern is a key characteristic for anti-inflammatory properties [91]. Depolymerisation can also be performed through a freeze-drying process, which can result in physical changes of some oligosaccharides within the LMWH molecule [117].

An alternative approach is to separate, isolate and identify the non-anticoagulant oligosaccharides of LMWH without prior chemical or enzymatic digestion. A major limitation in the separation of the structurally complex oligosaccharides of LMWHs is the lack of a high-resolution technique. Various analytical techniques, such as high-performance size-exclusion chromatography (HP-SEC) [175, 176], capillary electrophoresis (CE) [110, 113, 177] and reversed-phase ion-pair high-performance liquid chromatography (RPIP-HPLC) [118, 162], have

been used to separate intact LMWHs. HP-SEC has been used to separate the oligosaccharides of LMWHs based on their molecular weights. This technique can partially resolve the smaller mass (up to dp8; ~2680 Da), but unable to resolve the higher mass oligosaccharides (>dp8) [176]. Apart from this, structurally different oligosaccharides having the same or similar molecular masses cannot be separated using this technique. Lately, CE has been employed to separate LMWHs [110, 113, 177]. The major limitation of CE is its limited separation of high molecular weight oligosaccharides. A RPIP-HPLC method has been previously developed in our laboratory to separate oligosaccharides of LMWHs [118]. This method successfully resolved both the higher and lower mass oligosaccharides. However, it requires a non-volatile tetrabutylammonium hydroxide ion-pairing reagent to effectively separate the highly negatively-charged LMWH oligosaccharides. The presence of a non-volatile ion-pairing reagent in the isolated oligosaccharides interferes with the biological assays while investigating their anti-inflammatory and other pharmacological activities [178].

A widely used analytical technique for the separation of oligosaccharides based on their charge is ion-exchange chromatography (IC). Its application has only been demonstrated for the separation of enzymatically or chemically digested oligosaccharides of LMWHs [179]. For example, IC has been used to separate disaccharides (dp2; ~670 Da) or oligosaccharides of uniform size consisting of six disaccharide (dp12; ~4020 Da) units. However, an intact LMWH, such as enoxaparin, is composed of a complex heterogeneous mixture of oligosaccharides ranging from dp2 to dp24 (~670 to 8040 Da). Therefore, in this study, we aimed to develop an effective IC technique for the separation of heterogeneous oligosaccharides of enoxaparin without its prior enzymatic or chemical depolymerisation. Using this

technique, various oligosaccharides or a mixture of oligosaccharides were isolated and their approximate molecular weights were determined. An application of this technique was demonstrated by identifying IC-separated oligosaccharides with or without anticoagulant activity. Furthermore, for the first time the anti-inflammatory activities of non-anticoagulant and anticoagulant oligosaccharides obtained without prior modification of the parent LMWH were investigated.

3.3 MATERIALS AND METHODS

3.3.1 Materials

The sodium salt of enoxaparin (100 mg/mL; 10000 IU/mL) was purchased from Aventis Pharma (Sydney, Australia). Fondaparinux sodium (5 mg/mL) was purchased from GlaxoSmithKline (Melbourne, Australia). Sodium chloride and methanol (AR grade) was purchased from Sigma Aldrich (Sydney, Australia). The mobile phase was degassed under vacuum and filtered through nylon filter membranes (47 mm diameter, 0.2 µm pore size, Grace Davison, Rowville, Australia) before use. The heparin derived unsaturated oligosaccharide by enzyme cleavage (dp2 to dp26; ~670 to 8710 Da) were purchased from V-LABS, (Covington, LA, USA). The anti-factor Xa (AFXa) kit was from American Diagnostica (Stamford, CT, USA). The murine macrophage cell line (RAW 264.7), L-glutamine, gentamycin, phosphate buffer saline, trypsin-EDTA, and lipopolysaccharides (LPS) from *Escherichia coli* were all purchased from Sigma Aldrich (Sydney, Australia). Tissue culture media containing RPMI-1640 and fetal bovine serum (FBS) were purchased from Invitrogen (NY, USA). *N*-(1-Naphthyl)ethylenediaminedihydrochloride (NEDD), sulfanilamide (SULF) and analytical grade isopropanol were purchased from BDH Chemicals (Poole, England).

3.3.2 Ion chromatography

A Dionex ion chromatography (Sunnyvale, CA, USA) consisting of a GP50 gradient pump and LC30 thermal compartment was used throughout this work. A GM-3 gradient mixer was installed before the injection valve. The LC30 thermal compartment was set at a column temperature of 40ºC. UV detection at 232 nm was performed with a Waters 486 tunable absorbance UV detector (Milford, MA, USA). Instrument control and data acquisition were performed using Chromeleon® software. A 250 µL sample loop was employed unless otherwise specified. Separations were performed on a semi-preparative Dionex CarboPac PA100 column (250 mm, 9 mm ID). Sodium chloride (NaCl) eluent gradients were prepared through mixing of a 2 M NaCl solution and Milli-Q water via the GP50 pump. The 2 M NaCl stock solution was prepared and filtered offline. The optimised NaCl eluent gradient was: 0-70 min: gradient from 32-74% NaCl in Milli-Q water (0.64 to 1.48 M NaCl). Total flow rate of 2.0 mL/min was maintained.

A goal of this work was to collect the relevant oligosaccharides of the separated intact LMWH so they could be tested for their pharmacological activity and it was advantageous to be able to load a large amount of sample onto the column without affecting the separation performance. Therefore, a semi-preparative column was chosen for this work with a sample loop of 250 µL. This allowed 10 mg of enoxaparin to be loaded onto the column for each injection.

3.3.3 Assay performance

Reproducibility of the method was investigated by calculating the intra- (n=6) and inter-day precision (five consecutive days, n=30) using peak area from five chosen peaks with analysis of 50 mg/mL (n=6) enoxaparin. Mean intra- and inter-day

retention times (five consecutive days) were determined based on peak retention time with repeat analysis of 50 mg/mL enoxaparin. The method robustness was investigated by determining the intra- (n=6) and inter-day (five consecutive days, n=30) retention times of five different chosen peaks with repeat analysis of 50 mg/mL enoxaparin using one of the three Dionex CarboPac PA100 (250 mm, 9 mm ID) column lots (Lot No. 008-20-166 or 004-27-047 or 012-26-041) and one of the three Dionex chromatography systems (IC consisting of a GP50 gradient pump, LC30 thermal compartment and a Waters 486 tunable absorbance UV detector or Biocompatible HPLC system consisting of a HPG-3400RS binary separation pump, WPD-3000RS auto-sampler, TCC-3000RS column thermal compartment and UV-3000RS detector or a 3300 UHPLC consisting of HPG-3400RS binary separation pump, WPD-3000RS auto-sampler, TCC-3000RS column thermal compartment and VWD-3000RS detector).

3.3.4 High-performance size-exclusion chromatography

The HP-SEC was performed on a HPLC system consisting of a Prostar 230 solvent delivery module, a Prostar 335 DAD detector and a Prostar 410 autosampler (Varian, Melbourne, Australia). Data acquisition and instrument control were carried out using Star Chromatography Workstation®software. The HP-SEC analyses were performed with a 200 µl sample loop and a 20 µl sample injection volume. Detection was monitored at 232 nm. The HP-SEC separations were performed using a Superdex™ peptide 10/300 GL (300×10 mm) size-exclusion column (GE Healthcare Bio-Sciences, Uppsala, Sweden) with an average pore size of 13 µm. The pore size of this column allows efficient separation of oligosaccharides ranging from 100Da to 7000Da and it was therefore selected for the analysis of enoxaparin (approximate

molecular weight range 600Da to 7000Da). Isocratic elution of enoxaparin, oligosaccharide standards or IC-derived enoxaparin oligosaccharides was performed with a 0.3 M sodium sulfate eluent at a flow rate of 0.2 mL/min.

3.3.5 Desalting and recovery of enoxaparin oligosaccharides

Enoxaparin was fractionated using IC. For each run (n=3), 20 oligosaccharides of enoxaparin (approximately 2 to 15 mL each) were collected over the same elution period between 29 to 65 minutes. Our initial experiments showed that the presence of a high concentration of NaCl (more than 0.9% w/v) interfered with the anticoagulant and anti-inflammatory assays. Therefore, the IC-collected oligosaccharides of enoxaparin were desalted using methanol precipitation [180, 181]. This involved the oligosaccharides being concentrated using a rotary evaporator (Model-Laborota 4000/4001 efficient, Heidolph Instruments, Schwabach, Germany) operated at 42ºC and 120 rpm until salt crystals began to form. Further, the oligosaccharides were precipitated by the addition of anhydrous methanol (80% v/v) followed by centrifugation at 3000 rpm for 10 min (Sorvall, DJB Labcare, Newport Pagnell, UK). Samples were then kept overnight at 4ºC. The supernatant containing NaCl was carefully discarded and the remaining traces of methanol were evaporated using compressed air. The precipitated oligosaccharides were dissolved in 500 µL of Milli-Q water. The sodium content and the recovery of desalted oligosaccharides were determined using flame photometry and IC respectively.

For the determination of NaCl content, a stock solution of NaCl (1 mg of sodium/mL) was prepared by dissolving 2.54 g of NaCl in one litre of Milli-Q water. The stock solution was serially diluted to prepare different standard sodium solutions at concentrations of 5 to 50 ppm. The standard solutions (n=3) were introduced into a

low temperature, single channel flame photometer (Model-PFP7, VWR International, Murarrie, Australia) and emission intensities were measured at 589 nm. The concentration of NaCl in the oligosaccharides (n =3) was calculated using the standard sodium calibration curve. To determine the loss of oligosaccharides during the desalting process, the desalted oligosaccharides were re-analysed using IC under the same chromatographic conditions as described previously.

3.3.6 Determination of molecular weight

Enoxaparin (10 mg/mL) or oligosaccharide standards (dp2 to dp18; ~670 to 6030 Da) were diluted to 2 mg/mL with Milli-Q water and analysed using HP-SEC. To determine the approximate molecular weights of enoxaparin oligosaccharides, the retention time of each standard oligosaccharide was compared against the retention time of HP-SEC separated enoxaparin peaks. Twenty different IC-separated oligosaccharides of enoxaparin were collected, desalted and analysed using HP-SEC to determine their approximate molecular weights. Furthermore, the HP-SEC separated three oligosaccharides (dp4, dp8 and dp10; ~1340, 2680 and 3350 Da) of enoxaparin were collected and individual oligosaccharide was reanalysed by IC.

3.3.7 Analysis of anticoagulant activity

The anticoagulant activity of desalted oligosaccharides was determined by a modified low-volume microtitre plate AFXa assay as described previously [117]. Briefly, the solutions provided in the assay kit were prepared as per the manufacturer's instructions. Ten µL (1 mg/mL) of sample (enoxaparin or IC derived desalted oligosaccharide) and 25 µL of anti-thrombin III and factor Xa were incubated for 3 minutes at 37°C. Substrate FXa was added and the solution was mixed and incubated for further 10 minutes. The reaction was quenched using glacial acetic acid and placed

in Eppendorf tubes which were centrifuged (Hettich, Tuttlingen, Germany) at 12,000 rpm for 1 minute and contents carefully transferred to wells of a microplate using a micropipette. After shaking for 60 seconds to remove any air bubbles present, the intensity of the colour, which was inversely proportional to the anticoagulant activity of oligosaccharide, was read at 405 nm using a microplate reader (Model-680, Bio-Rad Laboratories, Hercules, CA, USA). Each sample was analysed for AFXa activity in triplicate. Oligosaccharides with high, moderate, low or no anticoagulant activities were analysed for their anti-inflammatory activity.

3.3.8 Determination of anti-inflammatory activity

3.3.8.1 *Cultivation of murine monocytic macrophage cell line (RAW 264.7)*

Activated macrophages produce nitric oxide (NO) which is implicated in promoting inflammation causing cellular injury and tissue dysfunction [182, 183]. The inhibitory effects of enoxaparin-derived oligosaccharides on NO production in RAW 264.7 cells were investigated. For initial growth, the cells were seeded into 75 cm^2 tissue culture flasks and grown to confluence in RPMI-1640 culture medium as described previously [182].

3.3.8.2 *Stimulation of cells and measurement of NO*

For subsequent experiments, the cells were seeded into 96-well tissue culture plates at a density of 5×10^5 cells/well and incubated for 24 hours at 37°C and with 5% CO_2. Immediately after incubation, the cells were stimulated with 20 µL of LPS (100 mg/mL) in the presence or absence of enoxaparin oligosaccharides (100 µg/mL) or fondaparinux (5 mg/mL). Cells were then incubated in serum-free media at 37°C, 5% CO_2 for another 24 hours and the production of NO was estimated using the Griess

assay [184]. Briefly, after 24 hours of incubation, the cell supernatant (100 µL) was transferred into a new 96-well microtitre plate followed by the addition of equal volumes (100 µL) of Griess reagents (SULF and NEDD). The plate was kept in the dark at room temperature for 10 minutes before measuring the production of NO.

3.4 RESULTS AND DISCUSSION

3.4.1 Development and validation of IC method

Enoxaparin is a highly sulfated polysaccharide. Moreover, in basic solution, the hydroxide moieties of oligosaccharides deprotonate to provide additional negatively charged moieties which are available for interaction with the stationary phase when performing ion-exchange separations. Several columns were considered and tested to determine their suitability. Initially, the IC separation of enoxaparin was investigated using a Dionex AS20 anion exchange column using an instrument generated hydroxide gradient (1 to 100 mM) and suppressed conductivity and UV detection at 232 nm. The ease of on-line eluent generation, potentially highly sensitive suppressed conductivity detection and on-line desalting would be advantageous. However, this approach resulted in long separation times (200 minutes) exhibiting poor resolution and peak shape defects even when mobile phases containing higher concentrations of hydroxide were prepared. The resultant poor separation can be attributed to the fact that the oligosaccharides of enoxaparin could not be satisfactorily eluted from the column using a hydroxide gradient. The use of alternative eluents (which would need to be prepared offline) would not be compatible with suppressed conductivity detection, negating any advantages in using this column.

The Dionex CarboPac series of columns are designed for separation of carbohydrates, in particular highly negatively charged oligosaccharides. Initial

experiments with Dionex CarboPac PA1, PA10 and PA100 analytical columns showed promising signs of fractionation using NaCl eluents. An advantage of the PA100 over PA1 is that lower salt concentrations are required to elute oligosaccharides from the column (most likely due to the slightly lower ion exchange capacity and increased cross linking in the stationary phase). This is important if desalting of collected fractions is required. Satisfactory results were achieved with the CarboPac PA100, and importantly this column is available in a semi-preparative scale. This would allow larger sample sizes of highly concentrated samples to be injected without loss of resolution. This is an important consideration as fraction collection was required for further anticoagulant and anti-inflammatory testing. Furthermore, the influence of pH on the separation of enoxaparin was investigated by adjusting the mobile phase pH with hydrochloric acid or sodium hydroxide. Improved resolution was observed when the separation was performed at pH 6 (no pH adjustment required) compared to pH 3 and pH 11. The injection loop volume was maximised to 250 µL without significant loss of efficiency or resolution. The enoxaparin sample was also diluted in water or normal saline to investigate matrix effects. Improved resolution occurred when the sample was diluted in water, possibly due to self-elution effects of the saline in the sample. Injection concentrations were also maximised in consideration of fraction collection. An upper concentration of 50% enoxaparin could be injected whilst maintaining optimal chromatographic performance. Hence, a CarboPac PA100 semi-preparative column was used for the separation of enoxaparin using the same chromatographic conditions as described previously. Under these conditions, enoxaparin was separated into more than 30 different peaks (Figure 3.2). There was no major difference in the chromatographic separation of enoxaparin with semi-preparative CarboPac PA100 or analytical PA10

column. However, with the semi-preparative column the sample volume injected was 25 times greater and consequently provided enough mass in each separated fraction to facilitate isolation of the various enoxaparin oligosaccharides.

3.4.2 Assay performance

The assay performance was investigated using five different chosen peaks of enoxaparin (Figure 3.2). The intra- (n=6) and inter-day (five consecutive days, n=30) relative standard deviation (RSD) for each peak area was <5% respectively. The intra- and inter-day retention time RSDs for each of five peaks were <0.6% (n=6) and <0.9% (n=5) respectively. One of the important objectives of this study was to collect various oligosaccharides of enoxaparin and test their anticoagulant and anti-inflammatory activities. Therefore, an excellent reproducibility of retention time exhibited by this method is of great importance; allowing the collection of relevant oligosaccharides and thereby minimising the possibility of misleading anticoagulant and anti-inflammatory assay results. Method robustness was investigated from five chosen peaks of enoxaparin using three chromatography systems and three CarboPac PA100 column lots. The intra- (n=6) and inter-day (over five consecutive days, n=30) retention time RSDs for each of the five peaks obtained using different chromatography systems and different column lots were <0.6% (n=6) and <0.9% (n=30) respectively. These results indicated that changes in the chromatography systems and column lots did not affect the retention time of selected enoxaparin's oligosaccharides.

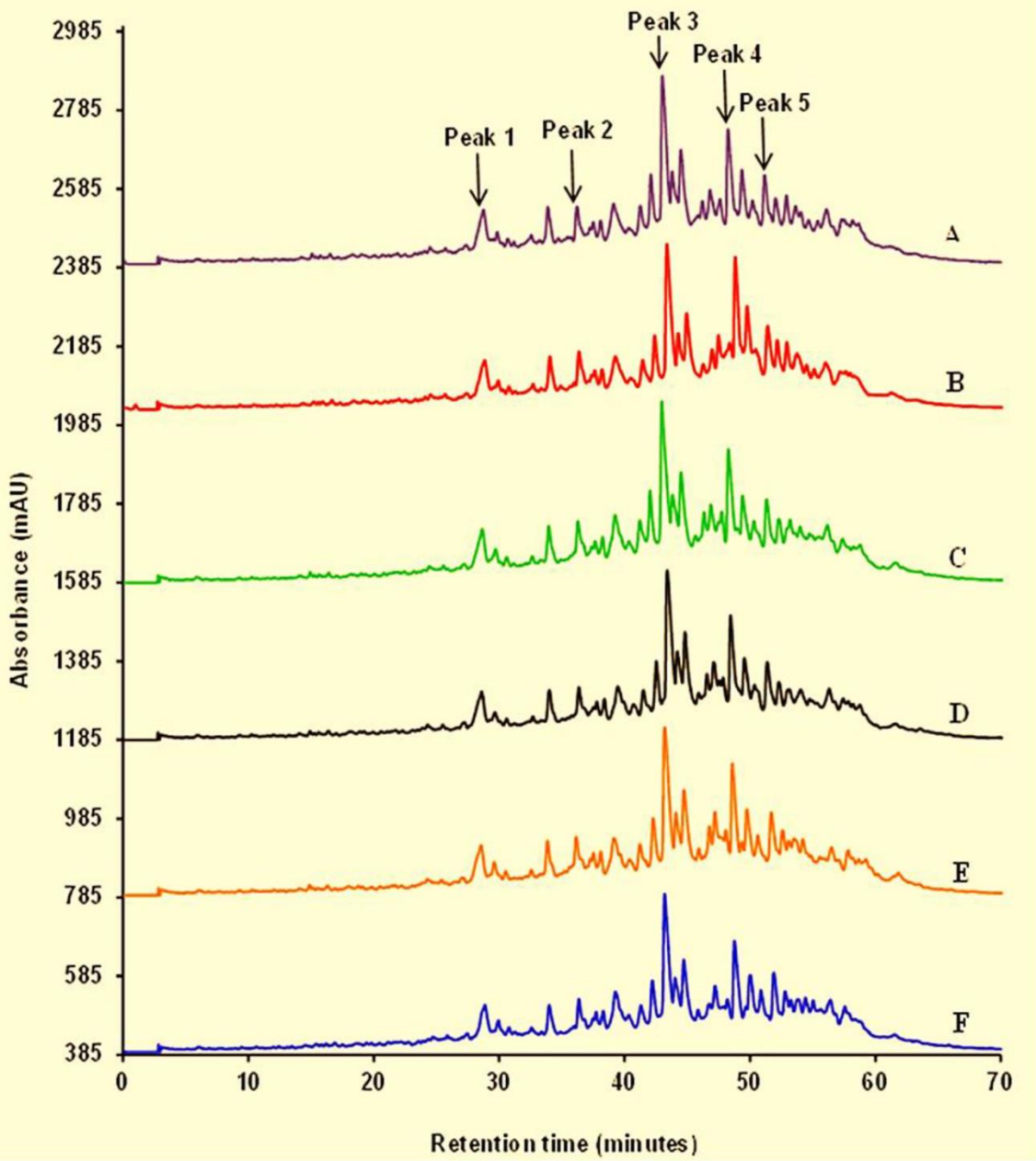

Figure 3.2 IC separation of enoxaparin **(A)**. Arrows indicate five chosen peaks of enoxaparin used for the determination of inter- and intra-day precision. Enoxaparin (50 mg/ml) was analysed five times on the same day **(B-F)**.

3.4.3 Determination of molecular weight

The oligosaccharide standards ranging from dp2 to dp18 and enoxaparin were analysed using HP-SEC (Figure 3.3A). Enoxaparin was partially resolved into 9 different peaks. The retention times of enoxaparin peaks were compared with the retention times of oligosaccharide standards. Based on this comparison, the approximate saccharide composition of enoxaparin oligosaccharides were assigned. The HP-SEC separation of analytes is based on the molecular size and therefore, the high molecular weight oligosaccharides (e.g. dp18) eluted earlier than the low molecular weight oligosaccharides (e.g. dp2).

Twenty different IC-derived oligosaccharides of enoxaparin were similarly analysed using HP-SEC. The approximate molecular weight of each oligosaccharide was determined by comparing the elution profiles of oligosaccharides against the HP-SEC separated enoxaparin peaks. The approximate molecular weights of IC-separated enoxaparin oligosaccharides are shown in Figure 3.3B. This technique successfully separated the smaller mass oligosaccharides (up to dp12) and partially separated the larger oligosaccharides of enoxaparin (up to dp20; ~6700 Da). It can be seen that the larger oligosaccharides eluted later during the IC salt gradient separation. As enoxaparin is constituted of sulfated oligosaccharides, the mechanism of this separation is based on the interaction of the sulfate groups with the oppositely charged stationary phase (ion-exchange interaction). As the oligosaccharides increase in size, so too does the degree of sulfonation.

Furthermore, three different oligosaccharides (dp4, dp8 and dp10) of HPSEC-separated enoxaparin were collected and analysed individually using IC (Figure 3.3C). Unlike HP-SEC, the IC successfully resolved dp4 and dp8 of enoxaparin into

more than five different peaks and dp10 into 4 distinct peaks. This highlights the resolving power of IC for the fractionation of structurally complicated oligosaccharides of enoxaparin. A LMWH such as enoxaparin, is highly sulfated linear polysaccharide composed of repeating disaccharide units of α-D-glucosamine and a hexuronic acid (α-L-iduronic or β-D-glucuronic acid) linked by 1→4 glycosidic bonds [128]. Multiple peaks of enoxaparin oligosaccharide having similar molecular weights (e.g. dp4) were observed in the IC chromatogram (Fig. 3c). A possible explanation for this could be the uneven distribution of the different pattern of sulfation along the enoxaparin's dp4 (two disaccharide units) chain. For instance, some disaccharide units have highly sulfated regions consisting of O-sulfation at position 2 of iduronic acid and N-sulfation at position 2 of glucosamine which are located at the non-reducing ends. On the other hand, some disaccharide units have under-sulfated regions consisting of non-sulfated iduronic acid residues and N-acetylation at position 2 of glucosamine. Therefore, the early eluted dp4 peaks potentially represent the under-sulfated disaccharide regions and the later eluted peaks represent the highly sulfated disaccharide regions of dp4 oligosaccharide of enoxaparin.

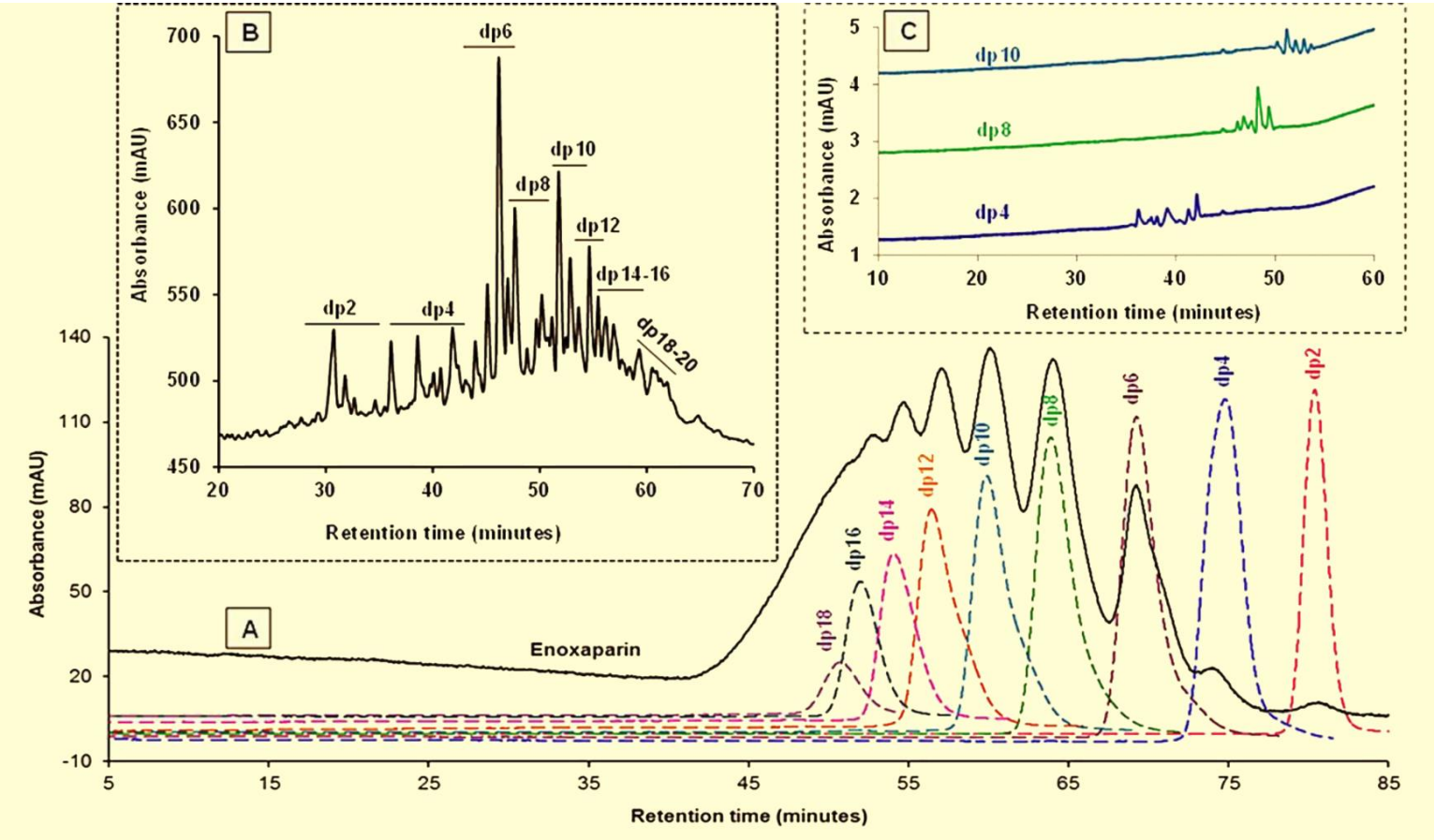

Figure 3.3 HP-SEC of enoxaparin (10 mg/mL) and oligosaccharides standards (2 mg/mL) **(A)**. IC of enoxaparin (25 mg/mL) with approximate saccharide compositions of oligosaccharides shown **(B)**. IC of HPSEC-collected dp4, dp8 and dp10 oligosaccharides of enoxaparin **(C)**.

3.4.4 Desalting and recovery of enoxaparin oligosaccharides

Our initial experiments showed that the presence of a high concentration of salt interferes with the anticoagulant and anti-inflammatory assays, and hence the desalting of oligosaccharides was crucial. Each collected oligosaccharide was precipitated using 80% w/v methanol. The precipitants were diluted with Milli-Q water and the concentration of NaCl was determined using flame-photometry. The concentration of NaCl in each oligosaccharide was found to be <0.9% w/v and was deemed satisfactory, as it is known that "normal" saline (0.9% w/v NaCl) is isotonic with body fluids and is used to dilute the oligosaccharides during anti-factor Xa analysis and to wash the cultured cells during determination of anti-inflammatory activity.

The recovery of desalted oligosaccharides was estimated using IC. The recovery was determined by calculating the differences in the peak areas of desalted oligosaccharide and oligosaccharide of enoxaparin eluted at the same time. Three oligosaccharides (dp4, dp6 and dp18; ~1340, 2010 and 6030 Da) corresponding to low, medium and high molecular weights were selected to determine their losses during the desalting process. The recoveries of dp4, dp6 and dp18 oligosaccharides were found to be 81%, 84% and 80% respectively suggesting the recovery is consistent across the wide molecular weight range. This technique allowed 10 mg of enoxaparin to be loaded onto the semi-preparative CarboPac PA100 column with each injection. This facilitated the collection of sufficient quantities of oligosaccharides for the investigation of their anticoagulant and anti-inflammatory activities. For instance, the quantity of IC-derived dp2 after desalting was found to be 0.63 mg/500 µL. Briefly, the approximate concentration of desalted dp2 oligosaccharide was

determined using a linear regression equation by constructing a calibration curve of the peak areas (obtained through IC) of dp2 standard (Sigma Aldrich, NSW, Australia) against their six different concentrations (0, 0.1, 0.5, 1, 1.5 and 2 mg/mL). This concentration (0.63 mg/500 µL) was sufficient to investigate its anticoagulant and anti-inflammatory activities for more than 20 and 5 times respectively.

3.4.5 Determination of anticoagulant and anti-inflammatory activity

The calibration curve for AFXa activity was plotted using six different enoxaparin concentrations (0-0.5 IU/mL) and the correlation coefficient (r^2) was greater than 0.987 (Figure 3.4). Four different oligosaccharides (dp2, dp6, dp12 and dp18) with no, low, moderate or high anticoagulant activities were tested for their anti-inflammatory properties. The anticoagulant activities of the selected oligosaccharides are shown in Figure 3.5. Disaccharide (dp2) of enoxaparin did not show any anticoagulant activity whereas the anticoagulant activities of dp6, dp12 and dp18 oligosaccharides were found to be approximately 1000, 5000 and 6000 IU/mL respectively. Pentasaccharide is the smallest sequence (dp5; ~1675 Da) required within the enoxaparin chain for the anticoagulant activity [5]. Disaccharides of enoxaparin are not long enough to bind AT III and hence did not exhibit any anticoagulant activity. The observed anticoagulant activities of dp12 and dp18 were five and six times higher than the activity of dp6. The possible explanation could be that dp12 and dp18 oligosaccharides are higher molecular weight chains than dp6 and therefore, they may contain more than a single AT III binding site.

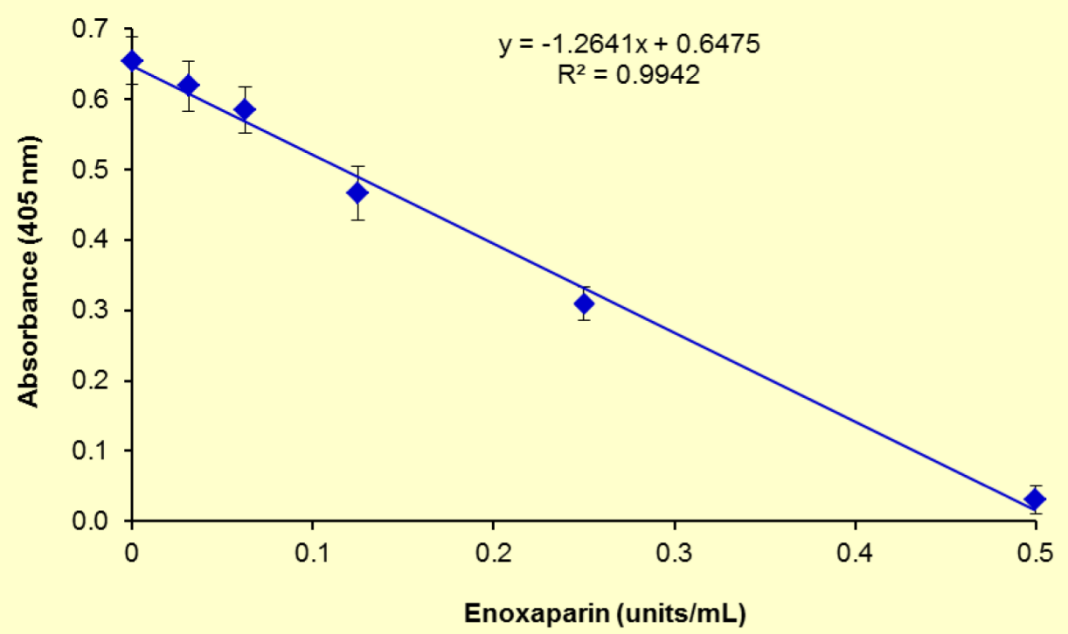

Figure 3.4 Calibration curve for the determination of AFXa activity using six different concentrations of enoxaparin. Sample (n=6) were analysed in triplicate and results are presented as mean ± SD.

To demonstrate an application of the newly developed IC-technique, the ability of IC-derived enoxaparin-derived oligosaccharides (dp2, dp6, dp12 or dp18) to inhibit NO production by LPS-activated macrophages was investigated by the Griess assay [184]. The concentration of NO present in the cell culture supernatant was calculated using the nitrite standard curve (0 to 100 µM). The calculated linearity (r^2) of the standard curve (0-100 µM) was greater than 0.990. Although, during inflammatory disorders (rheumatoid arthritis, inflammatory bowel disease, asthma etc.) the production of NO is increased in response to the secretion of cytokines and endogenous LPS [185, 186], it is not associated with the developmental features of these inflammatory disorders including asthma. The percentage of NO production inhibited by enoxaparin oligosaccharides is shown in Figure 3.5. Among the tested oligosaccharides, dp6 showed the highest inhibition of NO production (80%). Oligosaccharides with high anticoagulant activities (dp12 and dp18) were five times

less effective than dp6 in inhibiting the production of NO. Enoxaparin disaccharide did not exhibit any anticoagulant activity but interestingly it reduced the production of NO by 50%. To test whether the anti-inflammatory effects of enoxaparin are independent of its anticoagulant activity, the ability of an ultra-LMWH (fondaparinux) to inhibit NO production was tested. Unlike enoxaparin, fondaparinux is a synthetic pentasaccharide and does not contain non-anticoagulant oligosaccharides [187]. Fondaparinux, even at the concentration of 5 mg/mL, failed to inhibit NO production. These results are consistent with other published studies indicating that the anti-inflammatory effects of LMWHs are not dependent on its pentasaccharide sequence, which is essential for its anti-coagulant activity [13, 58, 109].

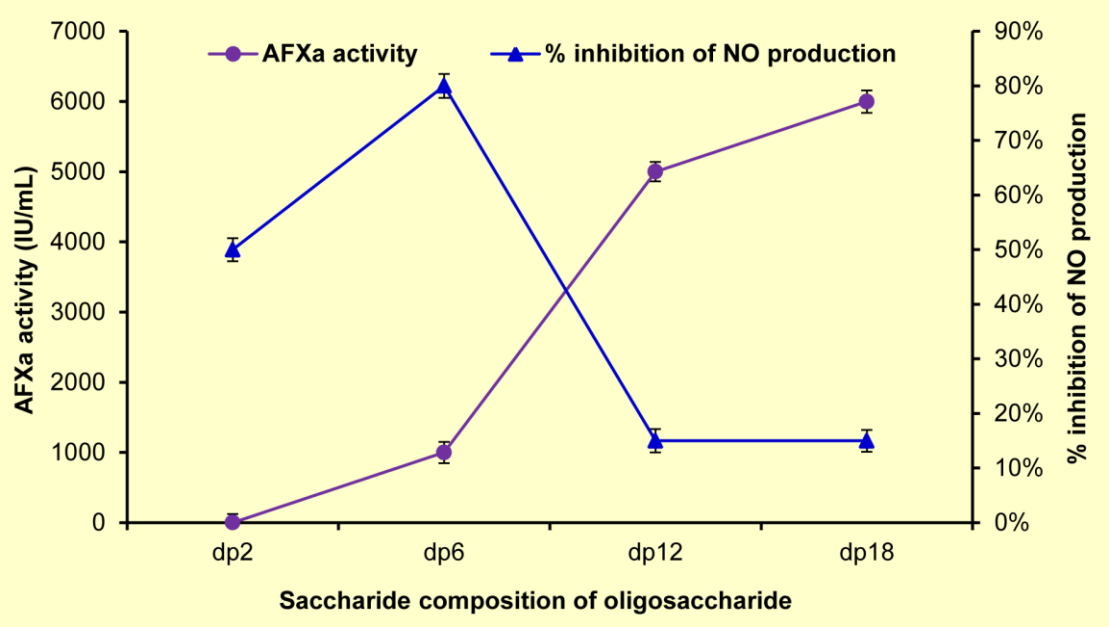

Figure 3.5 AFXa activity of IC-derived enoxaparin oligosaccharides and percentage inhibition of NO production by these oligosaccharides in LPS stimulated macrophages (RAW 264.7). Sample (n=3) were analysed in triplicate and results are presented as mean ± SD.

It is evident from experimental studies that the biological activity of a LMWH (e.g. enoxaparin) in a variety of biological disorders is independent of its anticoagulant property. These highly sulfated structurally unknown macromolecules are thought to play an essential role by interacting with important proteins involved in the pathogenesis of many disease states. To better understand these interactions it is imperative to identify and then characterize the non-anticoagulant oligosaccharides of LMWHs. Although the structure and biological significance of IC-collected enoxaparin oligosaccharides need to be elucidated in detail, these results clearly demonstrate that the newly developed IC method will provide a powerful tool for exploring the non-anticoagulant activities of enoxaparin-derived oligosaccharides.

3.5 CONCLUSION

In clinical practice, enoxaparin is widely used as an anticoagulant. However, to be employed as an anti-inflammatory agent, the risk of potential bleeding must be removed or minimised. In the present study, structurally complicated enoxaparin was successfully fractionated using a newly-developed IC method. An important application of this method was demonstrated by investigating the anti-inflammatory effects of its oligosaccharides. A number of studies have investigated the anti-inflammatory effects of intact enoxaparin. However, to our knowledge this is the first study demonstrating the anti-inflammatory effect of enoxaparin oligosaccharides obtained without prior chemical or enzymatic modification of the parent LMWH. Furthermore, this technique could potentially be used for structural characterization and identification of sulfation patterns of anti-inflammatory oligosaccharides of enoxaparin.

CHAPTER FOUR

Non-Anticoagulant Fractions of Enoxaparin Suppress Inflammatory Cytokine Release from Peripheral Blood Mononuclear Cells of Allergic Asthmatic Individuals

4.1 ABSTRACT

***Background*:** Enoxaparin, a low-molecular-weight heparin, is known to possess anti-inflammatory properties. However, its clinical exploitation as an anti-inflammatory agent is hampered by its anticoagulant effect and the associated risk of bleeding. The aim of the current study was to examine the ability of non-anticoagulant fractions of enoxaparin to inhibit the release of key inflammatory cytokines in primed peripheral blood mononuclear cells derived from allergic mild asthmatics.

***Methods*:** Peripheral blood mononuclear cells from allergic asthmatics were activated with phytohaemagglutinin (PHA), *concanavalin-A* (ConA) or p*horbol 12-myristate 13-acetate* (PMA) in the presence or absence of enoxaparin fractions before cytokine levels were quantified using specific cytokine bead arrays. Together with nuclear magnetic resonance analysis, time-dependent and target-specific effects of enoxaparin fractions were used to elucidate structural determinants for their anti-inflammatory effect and gain mechanistic insights into their anti-inflammatory activity.

***Results*:** Two non-anticoagulant fractions of enoxaparin were identified that significantly inhibited T-cell activation. A disaccharide fraction of enoxaparin inhibited the release of IL-4, IL-5, IL-13 and TNF-α by more than 57% while a tetrasaccharide fraction was found to inhibit the release of tested cytokines by more than 68%. Our data suggest that the observed response is likely to be due to an interaction of 6-*O*-sulfated tetrasaccharide with cellular receptor(s).

***Conclusion*:** The two identified anti-inflammatory fractions lacked anticoagulant activity and are therefore not associated with risk of bleeding. The findings highlight the potential therapeutic use of enoxaparin-derived fractions, in particular tetrasaccharide, in patients with chronic inflammatory disorders.

4.2 INTRODUCTION

Low-molecular-weight heparins (LMWHs) are a heterogeneous mixture of structurally complex oligosaccharides [188]. In clinical practice these molecules are widely used as anticoagulants but additionally, they are reported to possess anti-inflammatory properties [12, 189]. Enoxaparin, the most widely used LMWH, is known to inhibit T cell mediated release of multiple cytokines, such as IL-4, IL-5, IL-13 and TNF-α, involved in various inflammatory disorders including asthma [190]. A model for a chronic inflammatory disease and a potential target for an anti-inflammatory therapy is asthma, a complex multifactorial disorder of conducting airways. Its inflammatory pathology is characterised by reversible airway obstruction and airway hyper-responsiveness. It is estimated that approximately 300 million people worldwide suffer from asthma and it is associated with severe morbidity, and sometimes even mortality [18]. Inflammation in asthma is characterised by activation of T-helper type 2 (Th2) cells, production of immunoglobulin E (IgE) antibodies and eosinophilia [191]. Although T cells residing in the lungs (most of which are memory cells) are important for the local defence mechanism, excessive recruitment of T cells (mainly Th2 type) in the lungs, as well as their response to various allergens, is believed to be largely responsible for coordination of the inflammatory processes associated with allergic asthma [192]. In response to allergens, Th2 cells do not proliferate in the lungs but rather migrate into the lung from regional lymph nodes and

then produce various inflammatory cytokines, such as interleukin (IL)-4, IL-5, IL-9, IL-13 and tumor necrosis factor (TNF)-α, and chemokines, such as monocyte derived chemokine, in response to the specific allergen [23]. IL-4 and IL-13 are normally involved in the process responsible for the production of IgE antibodies. IL-4 and IL-5 enhance the survival of eosinophils, resulting in eosinophilic inflammation. IL-13 (and IL-9 to some extent) is reported to be important for bronchial hyper-responsiveness [193]. On the other hand, TNF-α sustains lung inflammation by increasing the recruitment of neutrophils and eosinophils in the airways [194].

Inhaled corticosteroids are the most effective drugs currently available for the treatment of asthma [195]. They effectively control asthma in the majority of patients. However, apart from the fact that the prevalence of steroid-resistant asthma is rising [143], corticosteroids are associated with a number of problems. For example, the symptoms appear soon after the treatment is stopped and with long-term treatment they increase the risk of osteoporosis in adults and reduced bone growth in children [126, 127]. Also, at recommended doses they have little or no effect on the process of asthma-associated lower airway remodelling [31]. An advanced understanding of the complex pathophysiology that drives this inflammatory disease has resulted in the development of new and emerging biological therapies, such as monoclonal antibodies, that are directed against a specific type of cytokine that is involved either in cell proliferation or the inflammatory response [196]. Apart from their cost and potentially severe side effects (such as hypersensitivity and susceptibility to various types of infections), monoclonal antibodies, at least for the treatment of asthma, have so far failed to meet initial theoretical expectations. It has been proposed that their limited clinical efficacy could be due to their inability to simultaneously inhibit multiple cytokines involved in the pathogenesis of asthma [197]. Therefore, the search

for safer, cheaper and effective drugs for the treatment of asthma continues. Among these drugs, LMWHs have attracted much interest in the field of inflammation.

Several experimental and clinical studies have shown the beneficial effects of LMWHs in various types of inflammatory disorders, including asthma [40, 146, 198]. The precise mechanism(s) behind the anti-inflammatory effects of LMWHs are yet to be fully explored. However, it has been proposed that because of their high negative charge they can interact and modulate the activity of a wide range of biological molecules, including various types of immune cells and pro-inflammatory cytokines [147]. As the recruitment and activation of T cells in the lung is believed to be one of the central events in the initiation, progression and persistence of asthma, drugs (e.g. enoxaparin) that can suppress T cell-mediated inflammatory responses can be considered a promising therapeutic strategy [199]. However, like any other LMWH, enoxaparin is composed of both non-anticoagulant and anticoagulant components, referred to as oligosaccharides. Its potential use as an anti-inflammatory agent could be problematic due to the inherent risk of bleeding caused by the presence of anticoagulant oligosaccharides [147]. This problem can potentially be resolved by separating the anticoagulant fractions (oligosaccharides responsible for anticoagulant effect) and non-anticoagulant fractions (oligosaccharides potentially responsible for anti-inflammatory effect). However, separation of such a complex polysaccharide is a long-standing problem [200]. Nevertheless, we recently developed a novel chromatographic technique that allows the separation of different oligosaccharides of enoxaparin [105]. In the current study we isolated chromatographically separated oligosaccharides of enoxaparin and investigated their anticoagulant effect, as well as their ability to inhibit the T cell mediated release of important cytokines before postulating the potential mode of action alongside the structure activity relationship.

4.3 MATERIALS AND METHODS

4.3.1 Materials

All chemicals and reagents, if not otherwise stated, were obtained from Sigma-Aldrich (NSW, Australia). The sodium salt of enoxaparin (100 mg/mL) was purchased from Aventis Pharma (NSW, Australia), deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA), anti-factor Xa (AFXa) kit was purchased from American Diagnostica (Stamford, CT, USA) and fetal bovine serum was purchased from Invitrogen (NY, USA).

4.3.2 Recruitment of participants

Five healthy (mean age: 33.8 years, range: 26-48 years; 4 males, 1 females) and ten allergic asthmatic subjects (mean age: 40.9 years, range 19-59 years; 4 males, 6 females) were recruited by invitation. The healthy volunteers were not suffering from any acute or chronic diseases and the asthmatic subjects were suffering from no other diseases apart from intermittent asthma exacerbated by allergies. Exclusion criteria were use of systemic or inhaled corticosteroids or any other immunomodulatory medications within two months prior to blood sampling. No information of either the use of other medications or forced expiratory volume (FEV1) was obtained from the recruited participants.

4.3.3 Ethics statement

The research protocol was approved by the Health and Medical Human Research Ethics Committee (Tasmania, Australia) Network (Approval number: H0013117). Written informed consent for the collection of blood samples was obtained from the recruited subjects.

4.3.4 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood

Whole blood (80 mL) from each subject was collected before PBMCs were isolated. Isolation of PBMCs was conducted by standard methods via density separation using 30 mL of blood aliquots layered onto 20 mL of Histopaque. Following centrifugation at 200g for 30 minutes at 20°C (Eppendorf; Model: 5810R), PBMCs were aspirated from the Histopaque/aqueous interface and centrifuged at 700g for 10 minutes. Cells were washed twice with serum-free media and resuspended in complete medium [RPMI-1640 supplemented with 2.0 mM L-glutamine, 10% fetal bovine serum and antibiotics (penicillin G and streptomycin)].

4.3.5 Separation and isolation of enoxaparin fractions

Chromatographic separation of enoxaparin was carried out using a previously described ion-exchange chromatography (IC) method [105]. Briefly, enoxaparin separation was performed on a semi-preparative Dionex CarboPac PA100 (250 mm, 9 mm ID, 8.5 µm) strong anion-exchange column (Thermo Fisher Scientific, NSW, Australia). Mobile phases were composed of Milli-Q water (A) and 2 M NaCl (B). The linear NaCl elution gradient (mobile phase B; 32 to 74%) was used over 70 minutes with a constant flow rate of 2.0 mL/minute. A column temperature of 40°C was combined with UV detection at 232 nm.

4.3.6 Collection and desalting of IC separated enoxaparin fractions

IC separated enoxaparin fractions were collected and desalted as described previously [105] with minor modifications. Briefly, collected fractions were concentrated on a miVac DNA centrifugal concentrator (Genevac Ltd, Suffolk, UK) at 40°C and subsequently desalted using PD MidiTrap G-10 columns (GE Healthcare Life

Sciences, Uppsala, Sweden). The desalted fractions were kept at 4°C until use. The concentration of IC separated fraction of enoxaparin was determined by constructing a calibration curve from the peak areas of LMWH standards against their known concentrations. The recovery of each desalted fraction concentrations was calculated using the differences in the peak areas of the desalted fraction and enoxaparin fraction eluted at the same time. Each desalted fraction was tested for its anticoagulant activity and its inhibitory effect on cytokine release from activated PBMCs.

4.3.7 Determination of NaCl content

The NaCl content in the desalted fractions of enoxaparin was determined using flame photometry as described previously [105]. Briefly, a stock solution of NaCl (1 mg of sodium/mL) was prepared by dissolving 2.54 g of NaCl in one litre of Milli-Q water. The stock solution was serially diluted to prepare different standard sodium solutions at concentrations of 5 to 50 ppm. The standard solutions (n=3) were introduced into a low temperature, single channel flame photometer (Model-PFP7, VWR International, Murarrie, Australia) and emission intensities were measured at 589 nm. The concentration of NaCl in the desalted enoxaparin fractions (n =3) was calculated using the standard sodium calibration curve.

4.3.8 Analysis of anticoagulant activity

The potentiating effect of IC-derived enoxaparin fractions on anti-thrombin III inhibition of activated factor Xa was determined using a previously described low-volume microtitre plate anti-factor Xa assay [116]. Briefly, a solution containing anti-thrombin III, FXa and IC-derived enoxaparin fractions was incubated for 3 minutes at 37°C. FXa substrate was added immediately and the solution was incubated for another 10 minutes. The reaction was quenched using glacial acetic acid and the

intensity of developed colour was spectrophotometrically measured at 405 nm (Multiskan Go, SkanIt software, Thermo Fisher Scientific).

4.3.9 Preparation of stock solutions for PBMC culture treatments

Stock solutions of enoxaparin fractions at 1 mg/mL were prepared in RPMI-1640 medium and filter sterilized through 0.2 µm pore size syringe filters (Pall Life Sciences, Victoria, Australia). Other stock solutions were prepared accordingly: 2.5 mg/mL of phytohaemagglutinin (PHA) in RPMI-1640 medium, stored at -20°C; 5 mg/mL of concanvalin-A (Con A) in RPMI-1640 medium, stored at -20 °C; 1 mg/mL of phorbol 12-myristate 13-acetate (PMA) in DMSO, stored at -20°C and 5 mg/mL of fluticasone propionate in ethanol, stored at 4°C.

4.3.10 Desulfation of enoxaparin fraction

4.3.10.1 *Complete desulfation*

A solution containing 8 mg/mL of enoxaparin fraction was subjected to acid hydrolysis for complete removal of sulfate groups as described previously [150]. Briefly, 1 mL of nitric acid was added to the fraction in a capped glass vial and the solution was heated at 80°C overnight before adding another 0.2 mL of hydrogen peroxide. The temperature was raised to 110°C for an additional 6 hours. The mixture was neutralised using 1 M sodium hydroxide and diluted with 4 mL of Milli-Q water. Finally 200 µL of this solution was further diluted to 4 mL.

4.3.10.2 *N-desulfation*

A solution containing 8 mg/mL of enoxaparin fraction was incubated at 50°C for 30 minutes in the presence of tetrahydrofuran (133 µL) and water (7 µL) for partial *N*-

desulfation as described previously [151] with minor modifications. The mixture was neutralised using 0.1 M sodium hydroxide. The resulting product was evaporated to dryness and precipitated by the addition of anhydrous methanol (80% *v/v*) followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was carefully discarded and samples were kept at 4°C overnight. Any traces of methanol were removed using a miVac DNA centrifugal concentrator and the remaining precipitants were dissolved in 1 mL Milli-Q water.

4.3.10.3 *Selective 2-O-/3-O-desulfation*

Selective 2-*O*-/3-*O*-desulfation of enoxaparin fraction was performed as described previously [151]. Briefly, the fraction (8 mg/mL) was dissolved in 0.1 M sodium hydroxide (200 μL) and then lyophilised to dryness. The residues were dissolved in Milli-Q water (0.5 mL) and the pH was neutralised by addition of acetic acid. Finally, the resulting mixture was precipitated using anhydrous methanol as described above and dissolved in 4 mL Milli-Q water.

4.3.10.4 *Selective 6-O-desulfation*

Selective 6-*O*-desulfation of enoxaparin fraction was performed as previously described [151]. Briefly, the fraction (8 mg/mL) was mixed with equal volumes (1 mL) of tetrahydrofuran (solvent) and *N*-methyl-*N*-(trimethylsilyl) trifluroacetamide (silylating agent). The mixture was incubated at 50°C for 9 hours, evaporated to dryness and precipitated using anhydrous methanol as described above and finally dissolved in 4 mL Milli-Q water.

Each desulfated fraction was tested for its inhibitory effect on PBMC-induced release of cytokines. After desulfation, free sulfate groups were removed using a

1000Da cut-off filter (Millipore, NSW, Australia) at 15000 rpm for 10 minutes. The concentrated supernatant was dissolved in 1 mL of Milli-Q water for further use.

4.3.11 PBMC culture

Cells were cultured in 24-well cell culture plates in complete medium at a concentration of 1×10^6 cells/mL/well. Cells were incubated with 1, 2.5, 5, 7.5, 10, 20 or 40 µg/mL of PHA for 72 hours to identify the optimum concentration for the release of TNF-α. To measure the effect of fractions on the release of cytokines, the cells were stimulated with 10 µg/mL PHA in the presence or absence of IC-derived enoxaparin fractions. The effect of fractions was compared to positive control after addition of 0.5 ng/mL fluticasone in the presence of 10 µg/mL PHA. The target specific effect of enoxaparin fraction on cytokine release was examined after stimulation of cells with either 10 µg/mL Con A or 5 ng/mL PMA. The time dependency of enoxaparin fraction was determined after addition of the fraction at different time points in the presence of 10 µg/mL PHA and vice versa. The effect of various desulfated enoxaparin fractions was investigated in the presence of 10 µg/mL PHA. After 72 hours of incubation (37°C, humidified 5% CO_2 atmosphere), culture supernatants from each well were collected and analysed for the determination of cytokine concentrations using flow cytometry.

Each cell supernatant containing cell stimulants and/or various enoxaparin fractions, desulfated enoxaparin fractions or fluticasone was prepared and analysed in triplicate.

4.3.12 Measurement of cytokine release

The amounts of IL-4, IL-5, IL-13 and TNF-α secreted into the cell culture supernatant were analysed using a cytometric bead assay (Cytometric bead array flex sets; BD Biosciences, NSW, Australia), which was performed according to the manufacturer's instructions. Briefly, supernatant from different treatments was incubated with the beads of interest for 3 hours in the dark, prior to washing, resuspending and transferring to 96 well plates. The samples were run on a flow cytometer (FACS Canto, Becton Dickinson, CA, USA) and gates were set to include only singlet populations of beads. The assay detection limit was stated by the manufacturer to be ≈ 3 pg/mL, with recovery rates of ≥ 80% of protein. Data was analysed by FACS Diva Software. Each sample was analysed in triplicate.

4.3.13 PBMC viability assays

The effect of enoxaparin fraction on cell viability after 72 hours of incubation was assessed using two methods, trypan blue dye exclusion assay and lactate dehydrogenase (LDH) release into culture supernatant. Both assays employed routinely used methods. Cytotoxicity of treatment was determined using the LDH in-vitro toxicology assay kit (Sigma-Aldrich, NSW, Australia), according to the manufacturer's instructions. Briefly, PBMC culture supernatants were centrifuged at 250g for 4 minutes. An aliquot containing 50 µL of cleared supernatant was mixed with 100 µL of a solution containing LDH assay substrate, LDH dye and LDH cofactor and incubated at room temperature for 20 minutes before the reaction was terminated by the addition of 15 µL of 1 N hydrochloric acid. Absorbance at 490 nm was measured spectrophotometrically using a plate reader (Spectra Max M2 microplate reader, Sunnyvale, CA). Each sample was measured in triplicate.

4.3.14 Nuclear magnetic resonance (NMR) analysis

4.3.14.1 *Saccharide information of enoxaparin fraction*

Samples for NMR analysis were prepared in 50 mM potassium phosphate buffer (KPO_4) and 99.9% D_2O. All experiments were carried out on a Bruker Avance III HD 600 MHz spectrometer using a TCI triple-resonance cryogenically cooled probe with z-gradients all controlled by the software Topspin™ 3.2 (Bruker Corporation, MA, USA). Spectra were recorded at 25°C. Characterisation of fraction was performed at 100 µM using 1D and 2D ^{1}H spectroscopy (TOCSY 120 ms, COSY, ROESY 500 ms) and 2D ^{13}C-^{1}H spectroscopy (HSQC, HSQCTOCSY 120ms) with standard Bruker pulseprograms.

4.3.14.2 *Putative binding of enoxaparin fraction to PHA*

Saturation Transfer Difference-nuclear magnetic resonance spectroscopy (STD-NMR) was used to assess the potential binding of enoxaparin fraction to PHA by using the stddiffgp19.3 pulseprogram from the Bruker library, incorporating suppression of the residual water resonance with a Watergate sequence. The method was validated using a sample of bovine serum albumin (10 µM), tryptophan and glucose (100 µM) following previously published guidelines [201]. STD build-up curves were observed with saturation times of 0.5, 1.0, 2.0 and 5.0 seconds, respectively, with a range of shaped pulse power levels from 30-60 dB. An optimum saturation of 2 seconds was chosen from the steeper portion of the build-up curve, and 32 dB of saturation pulse power with an on-resonance excitation pulse at -1 ppm and off resonance at 30 ppm. 128 transients were recorded in 8192 data points with a relaxation delay of 1.5 seconds. The interleaved spectra were processed and difference spectrum calculated using Topspin™ 3.2 incorporating the stdsplit macro function. The potential binding

of enoxaparin fraction to PHA was examined using a 500 µL sample of fraction (100 µM) and PHA (5 µM) prepared in D_2O buffered with 50 mM KPO_4 at pH 7.0. The above experimental design was repeated over a range of saturation times from 0.5-5 seconds at 32 dB saturation pulsepower with up to 2000 transients recorded.

4.3.15 Statistical analysis

Results are presented as mean ± standard deviation (SD) or as percentage change in the release of cytokines following different types of treatments, compared either to PHA, Con A or PMA only controls. Each donor blood sample was treated as control (cells + cell stimulant) as well as treatment (cells + cell stimulant + enoxaparin fractions). The statistical analysis was performed on the raw data using a total mean response from all the mean values of controls as well as treatments. Given the fewer number of observations, statistical significance were evaluated using non-parametric Wilcoxon-signed ranked test and Kruskal-Wallis test, where applicable. Mann-Whitney U tests were then utilised to compare inter-group differences in place of Dunnett's post-hoc analysis. A *p*-value of <0.05 was considered statistically significant.

4.4 RESULTS AND DISCUSSION

4.4.1 Cytokine release

The release of TNF-α from PBMCs of allergic asthmatics in the presence of different concentrations of PHA is shown in Figure 4.1. Concentration-dependent increase in the release of TNF-α was observed. The release of TNF-α was found to be 315.1, 1099.0, 1465.8, 2337.8, 2852.7, 2925.4 and 2534.6 pg/mL at 1, 2.5, 5, 7.5, 10, 20 and 40 µg/mL respectively. The concentration of PHA at 10 or 20 µg/mL was found to be

the most effective in releasing TNF-α from allergic asthmatic subjects. There was no statistical difference in the release of tested cytokine when 10 or 20 μg/ml of PHA was used. Therefore 10 μg/ml of PHA was used for subsequent experiments. The reduction of TNF-α levels at higher concentrations (40 μg/mL) is most likely due PHA-induced cell apoptosis which has been reported previously [159, 160]. We selected 72 hours as the incubation time period for all the experiments as previous studies has shown that 10 μg/mL of PHA is optimum to give an appropriate response for the cytokines of interest in 3-day cultures [202]. After 72 hour incubation with PHA, PBMCs from allergic asthmatic subjects released significantly higher levels of IL-4, IL-5, IL-13 and TNF-α than PBMCs of healthy controls (Figure 4.2A) with TNF-α showing the highest levels of 1652.6 pg/mL overall. The highest proportional change was observed for IL-5 in PBMCs from allergic asthmatic individuals, where its levels were increased by 98.7%.

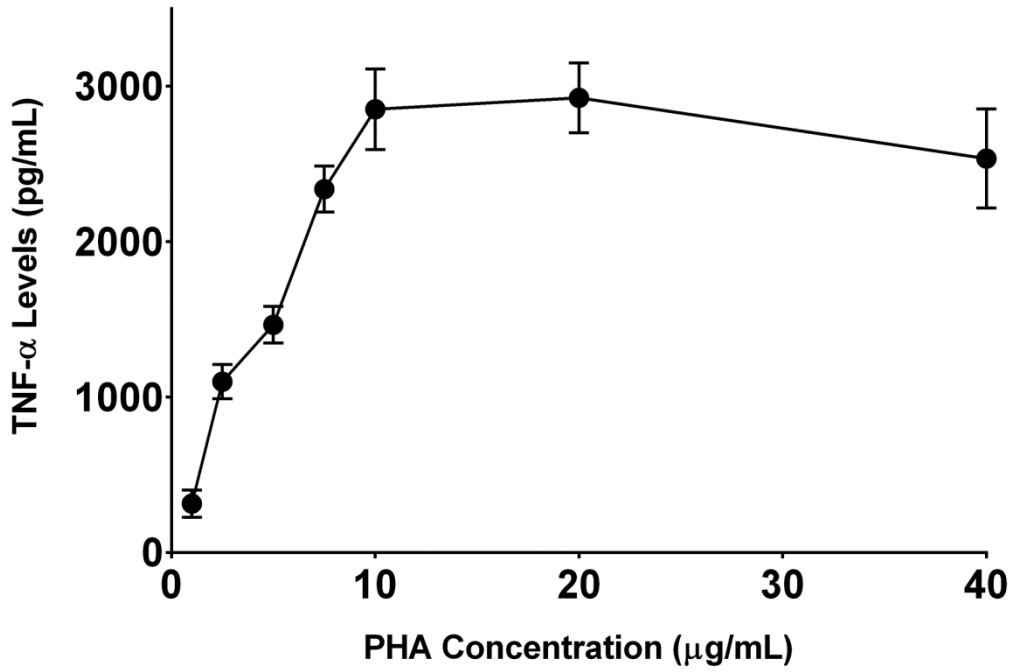

Figure 4.1 Concentration-dependent effect of PHA on the release of TNF-α. PBMCs from allergic asthmatic subjects (n=2) were stimulated with various concentrations of PHA (1 to 40 μg/mL) for 72 hours. Data is presented as mean ± SD.

4.4.2 Separation of enoxaparin

Beyond their well-established effect on blood-coagulation, LMWHs have been recognised for many other biological activities, including anti-inflammatory activity. Substantial evidence demonstrates that LMWHs could be effective for managing many inflammatory disorders including, asthma [41], ulcerative colitis [203] and lichen planus [204]. LMWHs have shown varying results, overall a clear benefit has been demonstrated, with enoxaparin being one of the most active LMWH [145, 146, 205]. Intact enoxaparin has shown to inhibit the release of IL-4, IL-5, IL-13 and TNF-α [190]. Nevertheless, the use of these complex macromolecules in inflammatory disorders is unlikely to progress because they harbour the inherent risk of bleeding complications due to their anticoagulant activities [161, 206]. To determine whether

the observed activity of intact LMWH is independent to its anticoagulant effect, enoxaparin was separated into different fractions with or without anticoagulant activity (Figure 4.2B-C). The approximate saccharide composition of each fraction ranging from two saccharide units (dp2; molecular weight ~600 Da) to 24 saccharides (dp24; molecular weight ~8000 Da) was previously determined using size-exclusion chromatography [105]. Each IC-derived enoxaparin fraction was collected, desalted and quantified. The concentration of NaCl in each desalted fraction of enoxaparin was found to be less <0.9% (*w/v*). This NaCl content was deemed acceptable since it is known that "normal" saline (0.9%, *w/v*, NaCl) is isotonic with body fluids and is used to dilute the fractions while performing the anti-factor Xa analysis and to wash the cultured cells during the determination of anti-inflammatory activity. Each fraction was tested for their effect on cytokine release from stimulated PBMCs of allergic asthmatic subjects.

4.4.3 Effect of IC-derived enoxaparin fractions on cytokine release

Since PHA induced the highest levels of TNF-α in the culture medium, this cytokine response was initially selected to test the effect of enoxaparin fractions in this cellular system. The isolated enoxaparin fractions modulated TNF-α release to different extents (Figure 4.2D). While the majority of fractions did not significantly change the levels of TNF-α, fractions 1 (disaccharide) and 4 (tetrasaccharide) significantly inhibited TNF-α release by 57.6 and 67.5% respectively. Interestingly, for the other two fractions (9 and 11) a trend towards increased TNF-α release was detected, which did not reach significance. To investigate the anti-inflammatory activity of enoxaparin in more detail we selected fractions 1 and 4 for further analysis.

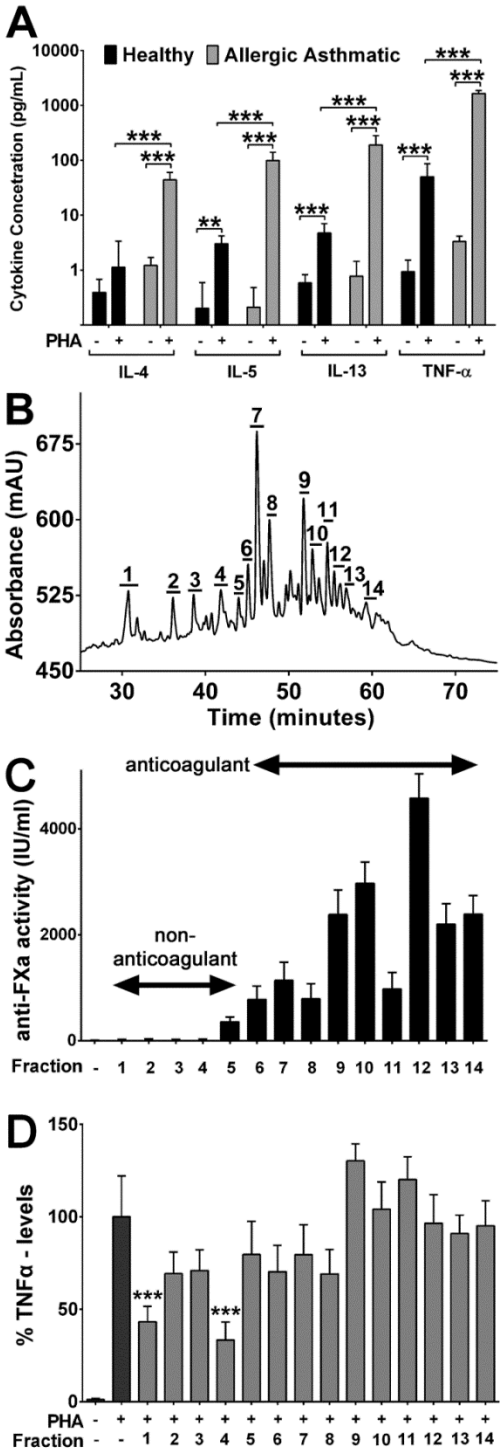

Figure 4.2 Effect of enoxaparin fractions on *ex-vivo* cytokine release. **(A)** PHA-induced release of cytokines (logarithmic scale) from PBMCs of healthy (n=5) and allergic asthmatic (n=10) subjects. Each sample was analysed in triplicate and the data is presented as the average of five and ten individual samples for healthy and allergic asthmatic subjects, respectively. Data is presented as mean ± SD. **(B)** Ion-chromatographic (IC) separation of enoxaparin, fractionated using a CarboPac PA100 semi-preparative column; a 32-74% NaCl gradient over 70 minutes; 2 mL/minute flow rate and a detection wavelength of 232 nm. The numbers indicate the area of all the fractions collected. Data represents a typical experiment out of five independent experiments. **(C)** Anticoagulant activity of IC-separated enoxaparin fractions. Data is presented as mean ± SD (n=3). Anticoagulant activity was observed from fraction 6 onwards. **(D)** Inhibition of TNF-α release by different fractions of enoxaparin. TNF-α was released by PBMCs of allergic asthmatic subjects (n=10) after *ex-vivo* stimulation with PHA. The relative percentile amount of each fraction (fraction 1 to 14) present in 500 µg/mL of intact enoxaparin was: 9.18%, 4.24%, 6.12%, 5.15%, 2.99%, 4.78%, 20.32%, 11.4%, 10.53%, 7.35%, 5.98%, 4.6%, 2.95% and 5.06% respectively. Data is presented as mean ± SD. ***p<0.001 versus the PHA-stimulated control.

4.4.4 Effect of fractions 1 and 4 on cytokine release

Using PBMCs from asthmatic subjects, the concentration dependent effects of fractions 1 and 4 were tested with regards to their inhibitory activity towards the release of IL-4, IL-5, IL-13 and TNF-α (Figure 4.3A-D). Both fractions significantly inhibited the release of all cytokines in a concentration-dependent manner. While fraction 1 showed maximal inhibition of cytokine release at 40 µg/mL, fraction 4 maximally reduced cytokine release at 20 µg/mL. Higher concentrations of fraction 4 did not result in a stronger inhibition. Instead a plateau of about 24-49% residual cytokine release compared to the PHA-stimulated control appeared to be the maximal possible level of inhibition by these fractions. Overall, inhibition by fraction 4 was greater than fraction 1, with maximum inhibition by 68.6%, 70.2%, 76.0% and 69.8% for IL-4, IL-5, IL-13 and TNF-α, respectively. The inhibitory effect of fraction 4 on TNF-α release in the presence of 20 µg/mL of PHA was also investigated and the percentage inhibition was found to be 67.14% which was not statistically significant to the observed inhibition seen with 10 µg/mL of PHA.

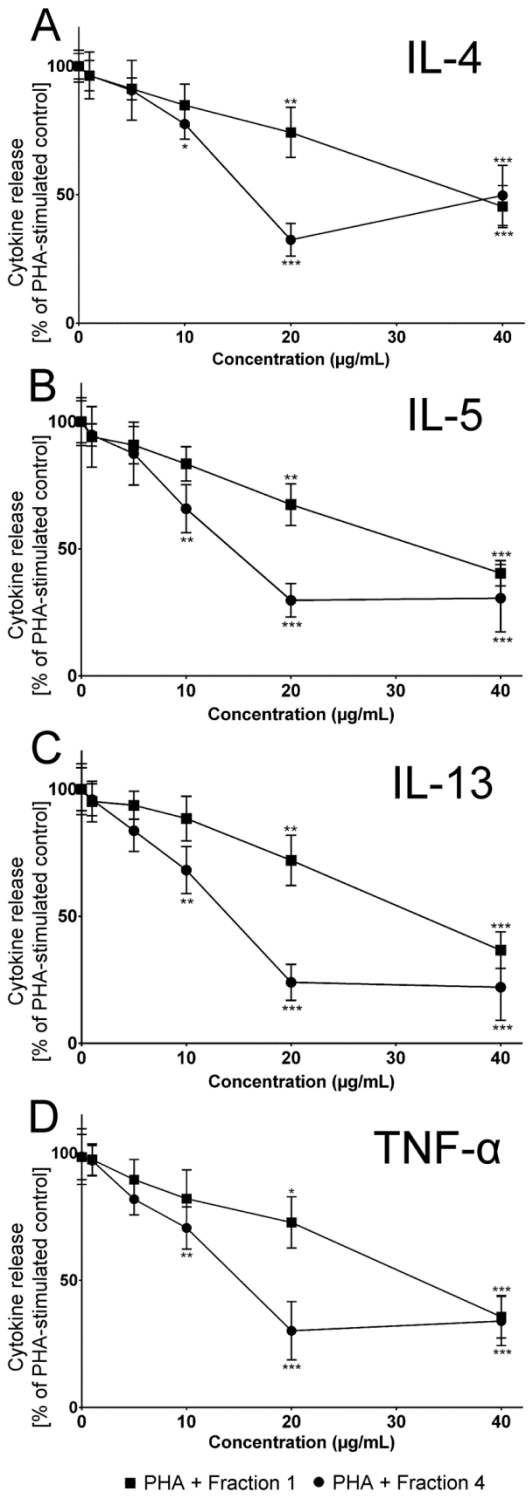

Figure 4.3 Concentration-dependent suppression of cytokine release by enoxaparin fractions. Inhibition of cytokine release by 0 to 40 µg/ml of enoxaparin fractions 1 and 4. Cytokines [IL-4 **(A)**, IL-5 **(B)**, IL-13 **(C)** and TNF- α **(D)**] were released by PBMCs of an allergic asthmatic subject (n=5) after *ex-vivo* stimulation with PHA. Data is presented as percentage of PHA-stimulated control. **p*<0.05, ***p*<0.01 and ****p*<0.001 versus PHA-stimulated control.

The observed inhibition of cytokine release by the two enoxaparin fractions 1 and 4 was comparable to the corticosteroid, fluticasone, one of the currently used agents in the management of asthma (Figure 4.4). There is evidence that the anti-inflammatory responses of heparins are known to be dependent on their chain lengths as well as sulfation pattern. For instance, it has been shown that the anti-proliferative activity exhibited by heparin requires oligosaccharide chains greater than six saccharides [90]. Moreover, *O*-sulfated heparin retains anti-proliferative activity and oversulfation at *O*-positions enhances this activity [89, 91]. Similarly, the observed differences in the inhibition of tested cytokines by different fractions could possibly be due to various degree of polymerisation, where a minimum of two and a maximum of four saccharide units are required to bind cellular receptors and exhibit inhibitory effects. The difference in the observed inhibitory effects of fraction 1 and fraction 4 could be high degree of sulfonation especially at *O*-positions in fraction 4 resulting in enhanced inhibition of cytokine release.

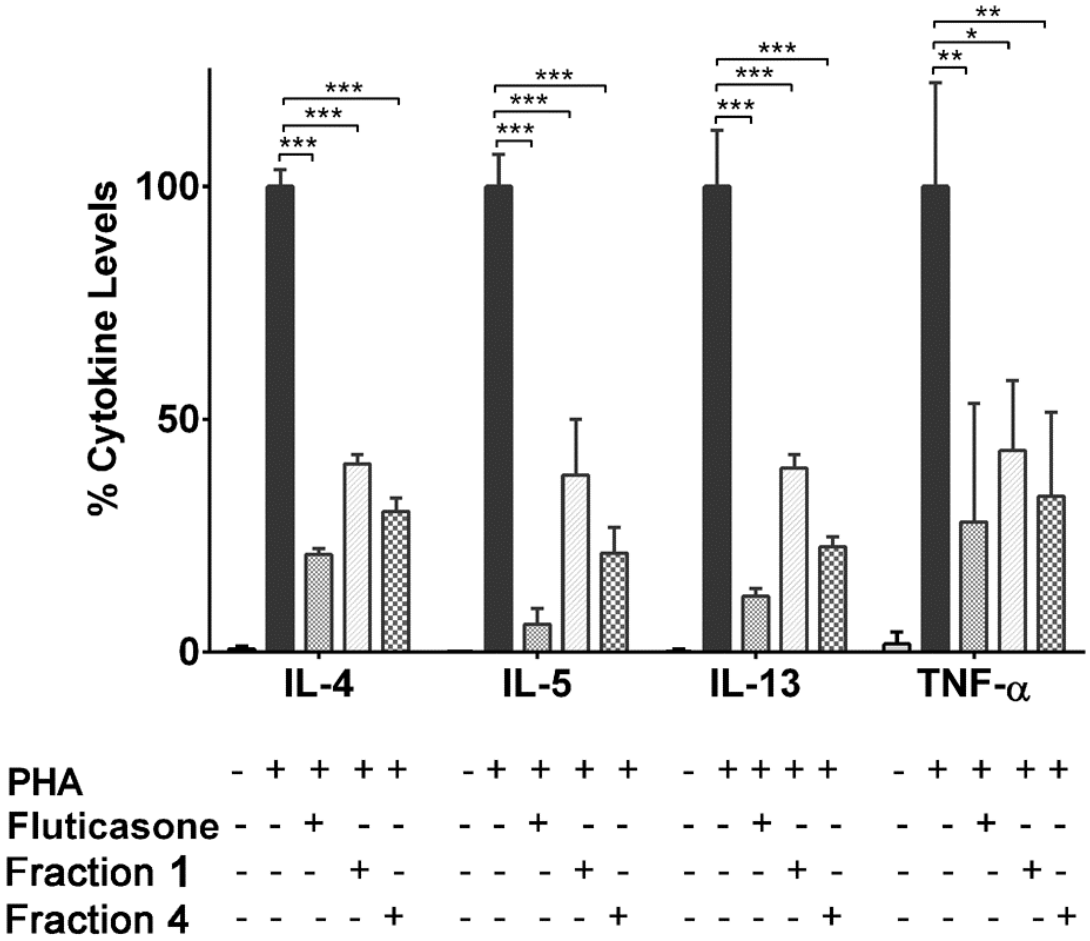

Figure 4.4 Comparison between enoxaparin fractions and fluticasone. Inhibition of cytokine release in the presence of fraction 1 (40 µg/mL), fraction 4 (20 µg/mL) or fluticasone (0.5 ng/mL). Cytokines (IL-4, IL-5, IL-13 and TNF- α) were released by PBMCs of allergic asthmatic subjects (n=10) after stimulation with PHA (10 µg/mL). Data is presented as percentage of PHA only control. **p*<0.05, ***p*<0.01 and ****p*<0.001 versus PHA only control.

4.4.5 Effect of fraction 4 on cellular viability and proliferation

To rule out that the observed inhibition of PBMC activation by fraction 4 arose as a consequence of cytotoxic effects or changes in cellular proliferation, we assessed cell viability in the presence or absence of PHA and/or fraction 4 by detecting LDH release into the cell culture supernatant (Figure 4.5A). Fraction 4 did not induce any increase of extracellular LDH while PHA, as expected, showed significant toxicity. This toxicity was not affected by co-incubation of PHA with fraction 4 (Figure 4.5A).

Consistent with the LDH-release results, no signs of cytotoxicity were detected in the presence of fraction 4 alone when using trypan blue exclusion as the toxicity readout (Figure 4.5B). In this case, only mild signs of toxicity by PHA could be detected, which when combined with fraction 4 was no longer significantly different to unstimulated PBMCs. Based on cell counts, PHA was found to induce significant proliferation of PBMCs. Fraction 4, on the other hand, showed no effect on cell proliferation when compared to either PHA or untreated control (Figure 4.5C).

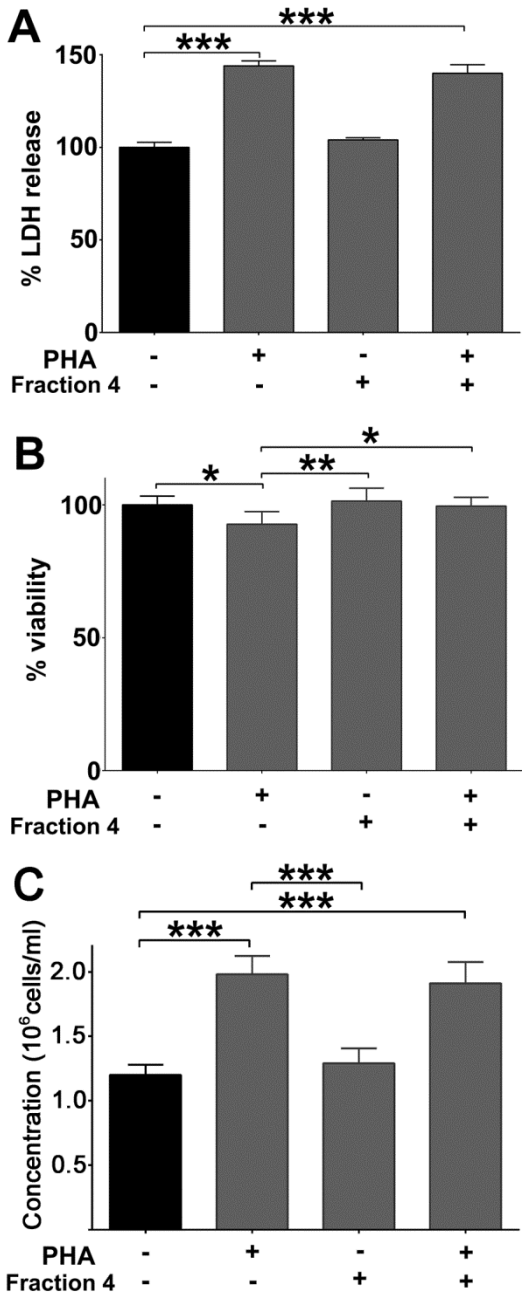

Figure 4.5 Effect of fraction 4 on cellular viability. (A) Viability of PBMCs is presented as mean % LDH release ± SD from allergic asthmatic subjects (n=3). Cells were incubated for 72 hours in the presence of PHA, fraction 4 or PHA + fraction 4. **p*<0.05 and ***p*<0.01 versus unstimulated control. **(B)** Viability of PBMCs from allergic asthmatic subjects (n=3) was determined by trypan blue dye exclusion test and is presented as % of viable cells remaining after 72 hours of incubation with PHA, PHA + fraction 4 or fraction 4 alone. Data is presented as mean ± SD. **(C)** Proliferation of PBMCs from allergic asthmatic subjects (n=3) was determined after 72 hours in the presence of PHA, fraction 4 alone or PHA + fraction 4. Cells were counted after incubation for 72 hours and expressed as million cells/mL. Data is presented as mean ± SD.

4.4.6 Time and target specificity of fraction 4

To gain a better understanding of the inhibitory mechanism of the fraction 4 of enoxaparin we investigated the time dependency of this process. When the PBMCs were treated with fraction 4 at different times after PHA stimulation, the inhibitory effect was lost after 10 minutes (Figure 4.6A). Maximal inhibition of TNF-α release was only observed if fraction 4 was added concurrently with PHA or at most 1 minute after PHA stimulation (Figure 4.6A). On the other hand, when PBMCs were pre-treated with fraction 4 up to 180 min before PHA stimulation, all time points showed effective inhibition of TNF-α release (Figure 4.6B).

To understand if this inhibitory effect of fraction 4 was restricted to a specific form of stimulation or molecular target, three different modes of activation were tested (Figure 4.6C). Consistent with prior results, fraction 4 significantly inhibited the release of TNF-α after stimulation of PBMCs with PHA. Similarly, a significant inhibition of TNF-α release was also observed after stimulation of PBMCs with Con A in the presence of fraction 4 (Figure 4.6C). Interestingly, the inhibitory effect of fraction 4 on TNF-α release was found to be completely absent when PMA was used to stimulate the PBMCs (Figure 4.6C).

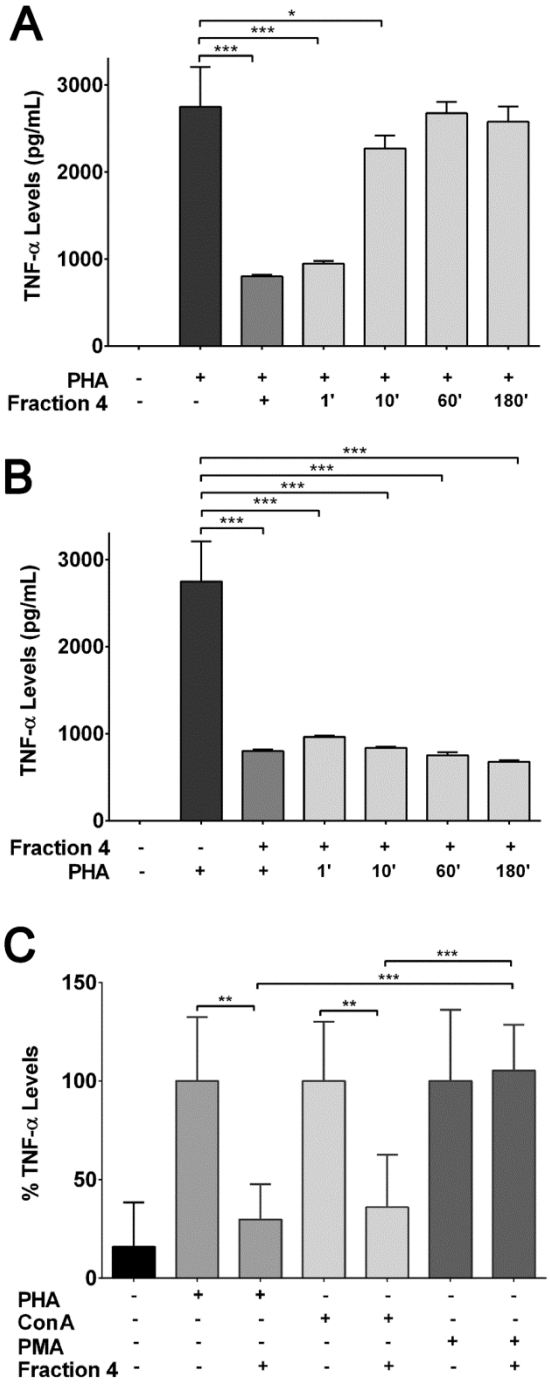

Figure 4.6 Time- and target-dependent inhibitory effect of fraction 4. Effect of fraction 4 on TNF-α release by activated PBMCs of allergic asthmatic subjects (n=3). **(A)** Addition of fraction 4 at different time points after the addition of PHA and **(B)** addition of PHA at different time points after the addition of fraction 4. Data is presented as mean ± SD. **p*<0.05 and ****p*<0.001 versus PHA only control. **(C)** Effect of fraction 4 on TNF-α release from activated PBMCs of allergic asthmatic subjects (n=3) after stimulation with either PHA (10 µg/mL), Con A (10 µg/mL) or PMA (5 ng/mL). Data is presented as mean ± SD. **p*<0.05, ***p*<0.01 and ****p*<0.001 versus PHA or Con A or PMA only control respectively.

4.4.7 NMR analysis

4.4.7.1 *Saccharide information of fraction 4*

NMR analysis was performed to identify the number of saccharide units present in fraction 4 of enoxaparin. A 2D ^{13}C-^{1}H multiplicity edited HSQC of fraction 4 specified the presence of four sugar units with single set of signals detected for each of the four sugar units (Figure 4.7). Therefore, fraction 4 of enoxaparin was confirmed to have a tetrasaccharide sequence.

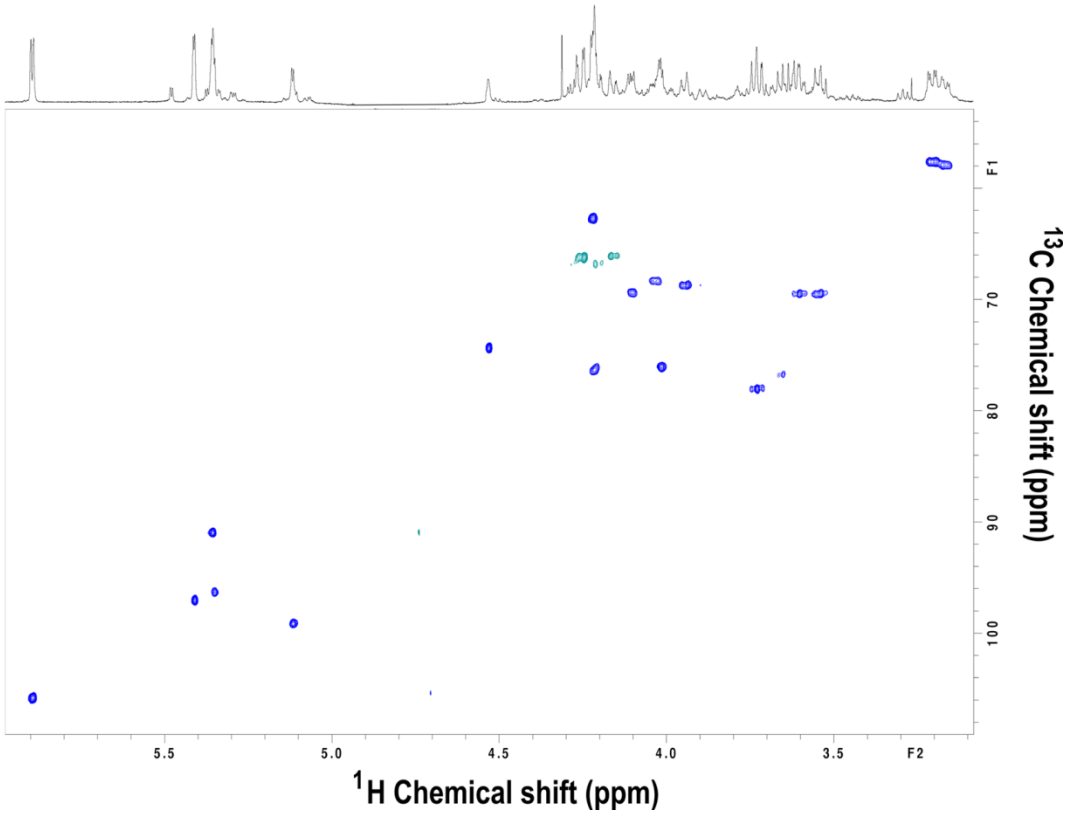

Figure 4.7 Saccharide Information of fraction 4. The 2D ^{13}C-^{1}H multiplicity edited HSQC spectrum for fraction 4. The blue contours represent signals from carbons with 1 or 3 attached protons and the cyan contours represent carbons with two attached protons, i.e. the CH_2 moieties of the two glucosamine units. This represents the presence of four sugar units with single sets of signals detected for each of the four sugar units and therefore, fraction 4 of enoxaparin was confirmed to have a tetrasaccharide sequence.

4.4.7.2 *Lack of PHA binding to fraction 4*

PHA is known to bind complex polysaccharides as well as the T-cell receptor [207]. Therefore, using STD-NMR we examined if fraction 4 of enoxaparin could bind to PHA directly to rule out an experimental artefact. Since direct binding of the two molecules could impair the ability of PHA to bind and activate the T-cell receptor we needed to confirm that the observed inhibition of T-cell activation by fraction 4 was not due to this potential *in-vitro* artefact of our experimental cell system. In the absence of any available small-molecule as a putative PHA binder, the STD-NMR experiments were validated using bovine serum albumin, tryptophan and glucose as described previously. As anticipated, a clear saturation as seen in spectrum B, reveals binding of tryptophan to bovine serum albumin, while in the same spectrum, no saturation of the glucose component confirmed its lack of binding (Figure 4.8).

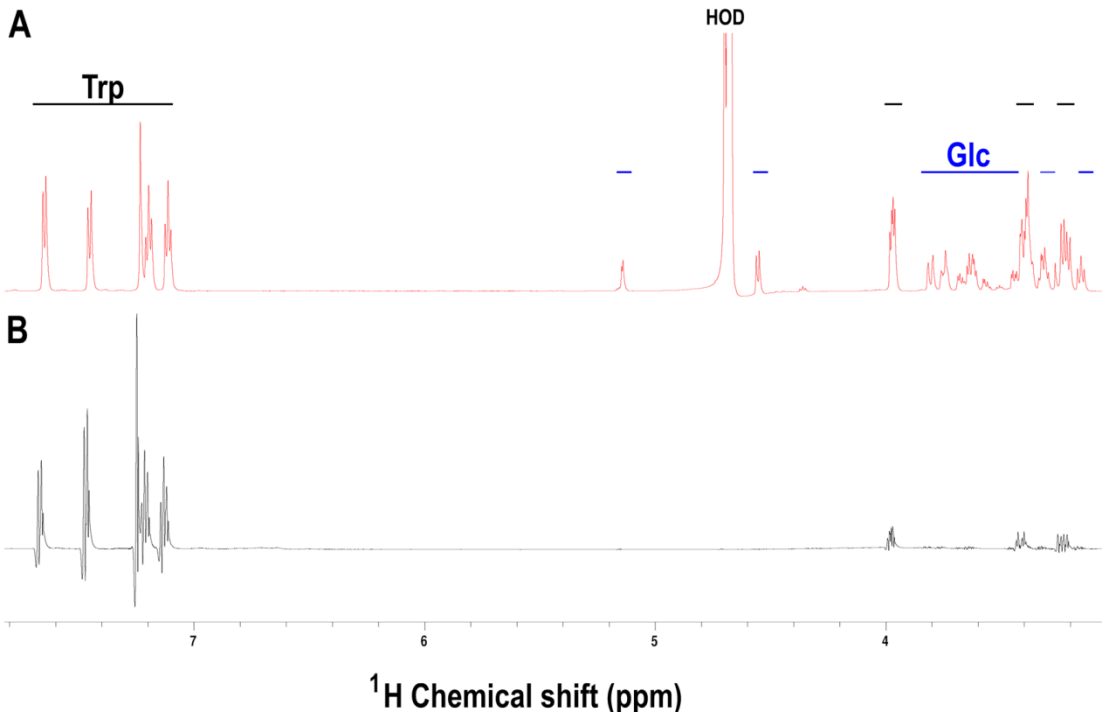

Figure 4.8 Control data for Saturation Transfer Difference (STD) spectroscopy. Two ^{1}H-1D spectra are presented for a solution of bovine serum albumin, L-Tryptophan (Trp) and Glucose (Glc). Spectrum **(A)** is the reference spectrum with attenuation of residual solvent

using pre-saturation at the solvent frequency. Spectrum **(B)** is the STD spectrum calculated from the difference of two spectra with excitation off-resonance (30 ppm) and on resonance (-1 ppm). The positive binding of Trp to BSA is indicated by the presence of signals that have obtained their excitation via the protein lattice. Glc does not bind and so does not register in the difference spectrum.

Under identical experimental conditions, PHA did not bind fraction 4 since no saturation of this fraction was detected after excitation (Figure 4.9). Spectrum A shows fraction 4 resonances from the ^{1}H-noespr1d experiment and spectrum B shows the only visible signals from excitation of PHA (Figure 4.9).

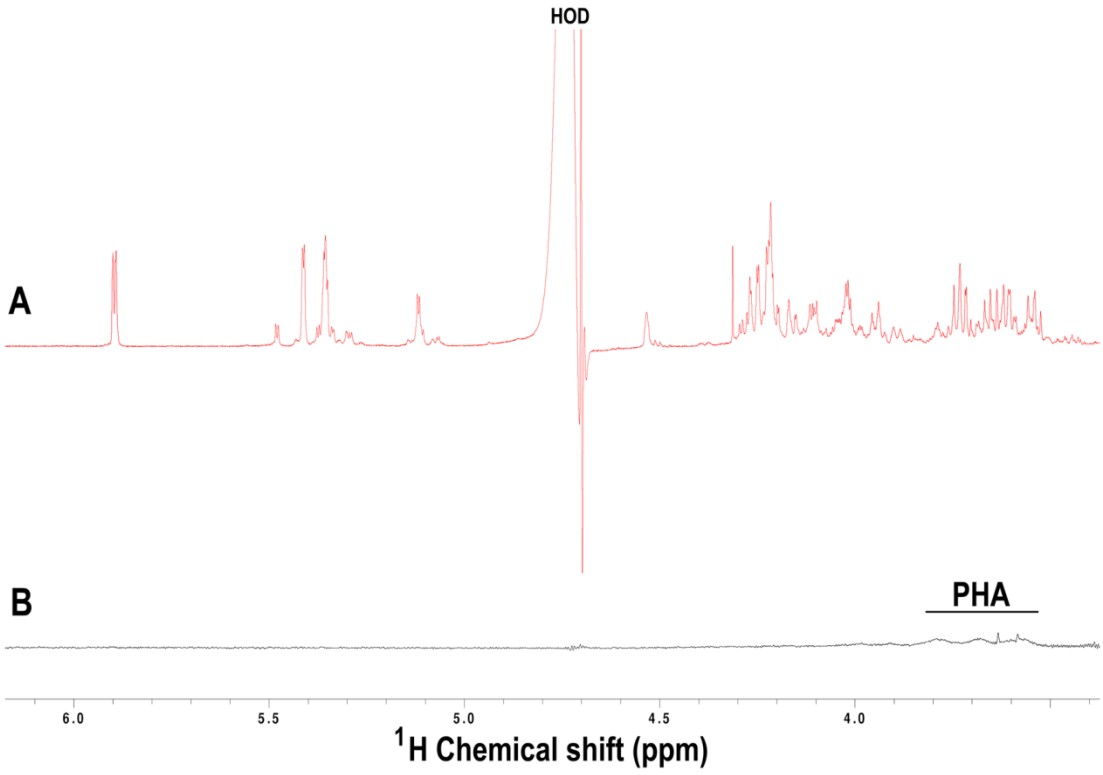

Figure 4.9 NMR analysis elucidating lack of binding of fraction 4 to PHA. Two ^{1}H-1D spectra are shown for the putative binding of fraction 4 to PHA. Spectrum **(A)** is the reference spectrum of fraction 4 and PHA obtained with suppression of residual solvent signal by pre-saturation at the solvent frequency. Spectrum **(B)** is the STD difference spectrum calculated from spectra with an on-resonance pulse at -1 ppm and off-resonance pulse at 30 ppm. Only the baseline of PHA signals can be observed in this trace. No binding of fraction 4 can be detected.

Among the potential mechanisms that could account for the anti-inflammatory effect of heparins, their interaction with a broad range of bioactive molecules involved in the process of inflammation, such as cytokines, growth factors, adhesion molecules, tissue-destructive enzymes and cytotoxic mediators has been proposed [13]. However, the mode(s) of action behind the observed anti-inflammatory effect of various types of heparins in disorders like asthma is poorly understood. It was previously suggested that the anti-inflammatory activity of heparins could be dependent on the inactivation of nuclear factor-kappa B, the inhibition of 1,4,5-inositol triphosphate-induced signalling or interaction with the CD11b receptor [40, 74, 146]. Here we describe for the first time, another mechanism by which enoxaparin fractions can inhibit a PBMC-mediated inflammatory response induced by receptor-activating ligands, such as PHA or Con A [208, 209]. Since the lectins, PHA and Con A are known to bind the CD3- and CD28-T cell receptor complex, as well as polysaccharides, the anti-inflammatory effects we observed by enoxaparin fractions could be a result of either: i) saturating the polysaccharide binding site of PHA and thereby blocking its binding to the T-cell receptor; ii) binding and blocking the PHA-binding site on the T-cell receptor; or iii) binding to an allosteric binding site on the T-cell receptor which prevents T-cell receptor activation. Since the NMR analysis demonstrated that the enoxaparin fraction 4 does not bind to PHA, a specific or allosteric binding to cellular receptor(s) complex is likely. This hypothesis is supported by our observation that the enoxaparin fraction 4 was unable to prevent the inflammatory response when PBMCs were stimulated with PMA, which activates protein kinase C (PKC) downstream of the T-cell receptor [210]. Finally, the general binding between cell surface T-cell receptors and PHA or Con A occurs rapidly after co-incubation [211]. For maximum activation and subsequent proliferation it has been

shown that the antigen receptor has to be engaged for at least 2 hours [211, 212] but a variety of immediate early genes such as interferon-gamma are induced after 30 minutes [213]. Based on the time-dependent inhibition of T-cell activation observed, our results strongly suggest that the potential mechanism by which enoxaparin fractions suppress the inflammatory response is by directly interacting with cell surface receptors and is covering different signalling pathways since both interleukins and TNF-α are supressed. It remains to be seen if this suppression is limited to activation through plant lectins or is extended to antigen-specific activation of the T cell receptor. The identification of specific unmodified enoxaparin fractions paves the way to map the specific binding site(s) involved in future studies.

4.4.8 Effect of desulfated fraction 4 on cytokine release

Since enoxaparin oligosaccharides are enriched with sulfate groups that are thought to be important for its anti-inflammatory activity, we examined the influence of sulfate groups by testing fraction 4 after desulfation of specific moieties with regards to their influence on the suppression of TNF-α release (Figure 4.10). It was observed that the inhibitory effect of fraction 4 was significantly reduced after complete and 6-*O*-desulfation of the fraction while *N*- and 2-*O*/3-*O*-desulfated fraction had no significant influence on the release of TNF-α. The inhibitory effect on the release of TNF-α after 6-*O*-desulfation was found to be reduced by more than 95%.

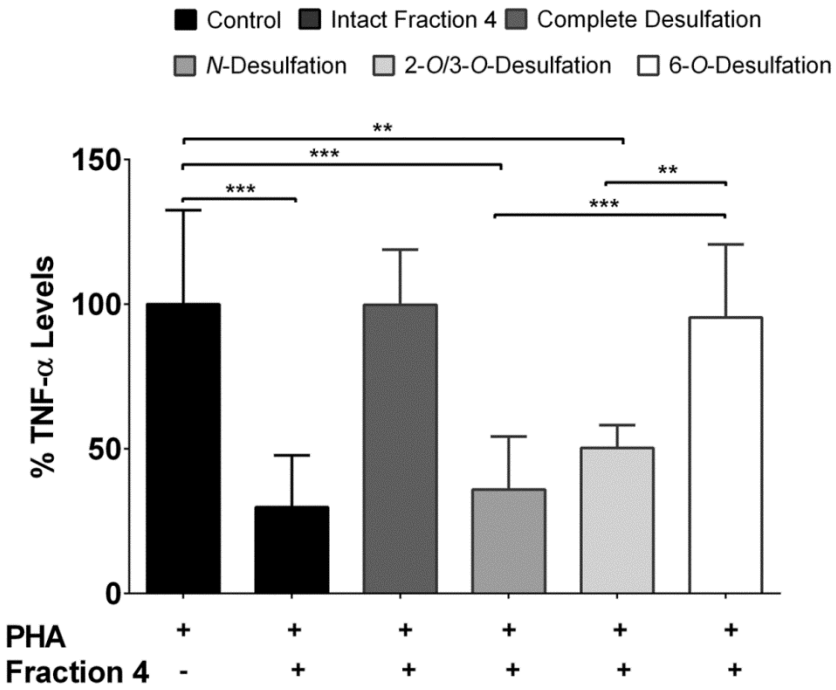

Figure 4.10 Effect of desulfation on anti-inflammatory activity of fraction 4. PBMCs of allergic asthmatic subjects (n=3) were stimulated with PHA in the presence of either completely desulfated, *N*-desulfated, 2-*O*/3-*O*-desulfated or 6-*O*-desulfated fraction 4 of enoxaparin. Data is presented as mean ± SD. **p*<0.05, ***p*<0.01 and ****p*<0.001.

To avoid such risk associated with the intact LMWHs, a range of attempts have been made to separate non-anticoagulant and anticoagulant fractions of oligosaccharides. One available approach is to chemically or enzymatically depolymerise the parent LMWH. However, depolymerisation can result in a structural modification of oligosaccharides, with a loss of biological activities exhibited by the parent molecules [115, 174]. For example, some oligosaccharides of LMWHs are heat-sensitive and can undergo chemical modification, especially removal of important sulfate groups, during the elevated temperatures of the depolymerisation process [116]. Furthermore, the presence and location of specific sulfate groups is critical to elicit anti-inflammatory effects (Figure 4.11) [49, 214-219]. For instance, sulfate groups at the 6-*O* position are essential for inhibiting leukocyte adhesion while sulfate groups at the 2-*O*/3-*O* and *N* positions are required for the inhibition of

chemotaxis or proliferation [49, 214, 216]. Therefore, the loss of any of these sulfate groups as a result of depolymerisation will impair a specific aspect of the anti-inflammatory response exhibited by the parent LMWH. Consequently, the protocol for separation, isolation and purification of oligosaccharide fractions from intact LMWH, as described here, represents a major step towards the identification of a sub-fraction that retains full anti-inflammatory activity of the parent molecule [190]. Our results demonstrate for the first time the requirement of 6-*O* sulfate groups for the suppression of PBMC activation while, removal of 2-*O*/3-*O*- and *N*-sulfate groups had little effect. Although selective desulfation procedures were performed using well established and validated analytical methods, a major limitation of the current study is that we did not investigate and confirmed the integrity of the desulfated fraction of enoxaparin.

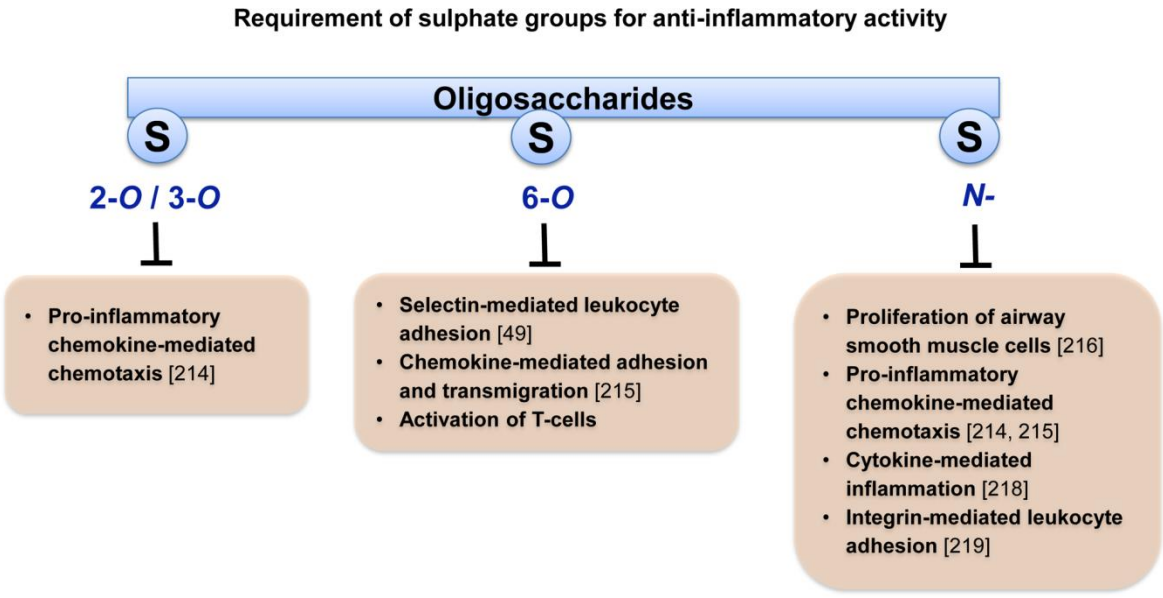

Figure 4.11 Schematic representation of the different sulfate groups required to elicit various anti-inflammatory responses.

4.6 CONCLUSION

In summary, the two identified fractions responsible for the anti-inflammatory effect of enoxaparin are composed of two and four saccharides. Therefore, the structure of these fractions eliminate the potential risk of bleeding as a minimum chain length of five saccharide residues (pentasaccharide) is required for the anticoagulant effect. Given the wide range of inflammatory mediators involved in the pathogenesis of asthma, supressing the release of multiple cytokines, as seen with the identified fractions, could provide significant clinical benefits. However, using more specific cellular models such as purified T-cells, anti-CD3/CD28 or intracellular staining of T cells, further work is required to determine whether enoxaparin fractions: i) act directly on T cell receptors and thereby, inhibit the release of T cell mediated cytokines and ii) retains the anti-inflammatory effect when an allergen other than PHA or Con A is used as a stimuli for the activation of T cells. The preliminary results obtained in the current *ex-vivo* study need to be confirmed by using pre-clinical *in-vivo* studies to determine dose-response relationships and to evaluate different formulation strategies. Due to their hydrophilicity and high molecular weight, intestinal absorption is likely to be very low. However, with respect to their use in asthma, a formulation for inhalation would not only deliver the fraction directly and selectively to its site of action, but it would also avoid systemic exposure to the drug, which overall makes this route of administration highly preferable.

CHAPTER FIVE

In-Vitro Suppression of IL-6 and IL-8 Release from Human Pulmonary Epithelial Cells by Non-Anticoagulant Fraction of Enoxaparin

5.1 ABSTRACT

Background: Enoxaparin, a mixture of anticoagulant and non-anticoagulant fractions, is widely used as an anticoagulant agent. However, it is also reported to possess anti-inflammatory properties. Our study indicated that enoxaparin inhibits the release of IL-6 and IL-8 from A549 pulmonary epithelial cells. Their release causes extensive lung tissue damage. The use of enoxaparin as an anti-inflammatory agent is hampered due to the risk of bleeding associated with its anticoagulant fractions. Therefore, we aimed to identify the fraction responsible for the observed anti-inflammatory effect of enoxaparin and to determine the relationship between its structure and biological activities.

Methods: A549 pulmonary epithelial cells were pre-treated in the presence of enoxaparin and its fractions. The levels of IL-6 and IL-8 released from the trypsin-stimulated cells were measured by ELISA. The anticoagulant activity of the fraction responsible for the effect of enoxaparin was determined using an anti-factor-Xa assay. The fraction was structurally characterised using nuclear magnetic resonance. The fraction was 2-*O*, 6-*O* or *N*-desulfated to determine the position of sulfate groups required for the inhibition of interleukins. High-performance size-exclusion chromatography was performed to rule out that the observed effect was due to the interaction between the fraction and trypsin or interleukins.

Results: Enoxaparin (60μg/mL) inhibited the release of IL-6 and IL-8 by >30%. The fraction responsible for this effect of enoxaparin was found to be a disaccharide

composed of α-L-iduronic-acid and α-D-glucosamine-6-sulfate. It (15μg/mL) inhibited the release of interleukins by >70%. The 6-*O* sulphate groups were responsible for its anti-inflammatory effect. The fraction did not bind to trypsin or interleukins, suggesting the effect was not due to an artefact of the experimental model.

Conclusion: The identified disaccharide has no anticoagulant activity and therefore eliminates the risk of bleeding associated with enoxaparin. Future *in-vivo* studies should be designed to validate findings of the current study.

5.2 INTRODUCTION

Asthma, a respiratory inflammatory disorder, affects millions of people around the world and its prevalence has increased markedly in recent years [144]. The pathogenesis of asthma is complex, involving different cell types, such as T-cells, neutrophils, mast cells, eosinophils, macrophages and epithelial cells of the lung [220]. Epithelial cells and macrophages act as the first-line of defence before the migration and recruitment of other types of inflammatory cells into the lungs upon exposure to various foreign particles, including allergens [221, 222]. Among various pro-inflammatory mediators, interleukin (IL)-6 and IL-8 are released by the pulmonary epithelial cells in response to their damage or stress caused by a variety of foreign particles [223]. IL-6 is a pro-inflammatory cytokine and its involvement in various immuno-regulatory effects, such as increased IL-4 production during Th2 differentiation, increased Th17 differentiation, decreased Th1 differentiation and increased IL-4 dependent synthesis of immunoglobulins, causes further damage to the lungs [224, 225]. IL-8, a potent chemotactic agent, facilitates the migration of

neutrophils and T-cells, and priming of eosinophils [226]. These immune cells can then cause extensive tissue damage and prolong the inflammatory phase.

Corticosteroids are currently used as first line agents for the treatment of asthma and are effective in supressing the release of various inflammatory mediators involved in the pathogenesis of disease. However, they are associated with severe side effects, especially with their long-term use in children, and there has been a consistent rise in the prevalence of difficult to treat corticosteroid-resistant asthma [143, 227]. More recently, monoclonal antibodies have been developed to treat various immunological disorders, including asthma. For example, omalizumab, a humanised monoclonal-immunoglobulin specific antibody, is the only biological approved for the treatment of severe, uncontrolled and steroid-resistant asthma. However, apart from its high cost and inconvenient route of administration, it is associated with serious side effects, such as anaphylactic reactions as well as occurrence of cardiovascular and cerebrovascular adverse effects [196, 228]. Therefore, there is a need for the development of novel therapeutic modalities for management of asthma.

Enoxaparin, a well-known anticoagulant drug, has recently attracted much research interest for its anti-inflammatory properties. This low-molecular-weight heparin (LMWH) belongs to the family of glycosaminoglycans and is composed of various fractions, also known as oligosaccharides, of unfractionated heparin (UFH) [131]. Although enoxaparin is commonly used for the prophylaxis of deep vein thrombosis [229], clinical studies have implicated its usefulness for the management of various inflammatory conditions, including asthma [36, 41, 166]. Like UFH and other LMWHs, enoxaparin is also composed of a mixture of anticoagulant and non-anticoagulant oligosaccharides [153]. Unfortunately, given the increased risk of

bleeding associated with anticoagulant oligosaccharides of enoxaparin, its use is not indicated for the management of inflammatory disorders [230]. However, clinical studies have indirectly indicated that the observed anti-inflammatory effects of heparins (UFH and LMWHs) are because of the presence of non-anticoagulant oligosaccharides and, hence, independent of their anticoagulant effect [12, 147]. One potential way to avoid the bleeding complications associated with heparins when used for the management of inflammatory conditions is to identify the non-anticoagulant oligosaccharides responsible for their anti-inflammatory effects. The current approach to obtain the oligosaccharides is to perform depolymerisation of heparins by chemical or enzymatic methods. However, a chemical or enzymatic depolymerisation results in the structural modification of oligosaccharides and it has been demonstrated that certain biological functions of the parent heparin could indeed be removed by the depolymerisation process [115, 173, 174]. Some oligosaccharides in heparins are heat sensitive and can undergo chemical modification, especially desulfation, with the elevated temperatures of the depolymerisation process [116]. An oligosaccharide's sulfation pattern is a key determinant for its anti-inflammatory properties. Depolymerisation can also be performed through a freeze-drying process; however, freeze-drying results in physical changes of some oligosaccharides within the heparin molecule [117].

An alternative approach is to separate, isolate and identify the non-anticoagulant oligosaccharides of heparins without their prior chemical or enzymatic digestion. However, a major limitation in the separation of heparins is the lack of a high resolution technique as they are highly negatively charged and structurally complicated compounds [113, 118]. Nevertheless, we have recently developed a novel ion-exchange chromatographic (IC) technique that can effectively separate the

anticoagulant and non-anticoagulant oligosaccharides of enoxaparin without their structural modification [105]. Since our study indicated that enoxaparin can inhibit the release of two key pro-inflammatory mediators involved in the pathogenesis of asthma (IL-6 and IL-8), we first identified the IC-derived fraction responsible for the observed inhibitory effect of enoxaparin and then characterised its structure, as well as determined the specific position of sulfate groups required for the inhibition of IL-6 and IL-8 release.

5.3 MATERIALS AND METHODS

5.3.1 Materials

A549 human pulmonary epithelial cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Enoxaparin was obtained from Aventis Pharma Ltd. (NSW, Australia). UFH was purchased from Hospira Pty. Ltd. (Victoria, Australia). Sodium chloride, tetrahydrofuran, sodium hydroxide, *N*-methyl-*N*-(trimethylsilyl) trifluroacetamide, methanol, acetic acid, Ham's F12K medium, antibiotics (penicillin G and streptomycin), trypsin-ethylenediaminetetraacetic acid (EDTA), trypsin, thrombin, enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and IL-8, trypan blue exclusion assay kit, lactate dehydrogenase (LDH) activity assay kit, and potassium phosphate monobasic and dibasic were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Fetal bovine serum, IL-6 and IL-8 recombinant human proteins were obtained from Invitrogen (Grand Island, NY, USA). Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

5.3.2 Human pulmonary epithelial cell (A549) culture

For initial growth, the A549 cells were cultured in 75 cm^2 tissue culture flasks (Corning, NL, Mexico) and grown to confluence in complete medium [Ham's F-12 Kaighn's Modification medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 µg/mL antibiotics (penicillin G and streptomycin)] and incubated at 37°C in a humidified 5% CO_2 atmosphere as described previously [231].

5.3.3 Preparation of stock solutions

Stock solutions of enoxaparin at 1 mg/mL were prepared in serum free medium (Ham's F12K) and filter sterilized through 0.2 µm pore size syringe filters (Pall Life Sciences, Victoria, Australia). Other stock solutions were prepared accordingly: 1.0 µg/mL trypsin (A549 human pulmonary epithelial cell stimulant) in Ham's F12K, stored at -20 °C.

5.3.4 Preparation of epithelial cell culture supernatants

At confluence, the cells were detached from the flasks using trypsin-EDTA solution and re-seeded in 24-well cell culture plates at a density of 1×10^5 cells/well. The cells were incubated for 24 hours and grown to confluency in serum free medium. After reaching confluency, the cells were exposed to 0.003 µg/mL of trypsin in the presence of either Ham's F12K medium (negative control) or different concentrations (5, 10, 20, 40, 60, 80 and 100 µg/mL) of enoxaparin. After 24 hours of incubation (37°C, humidified 5% CO_2 atmosphere), cultures were centrifuged, and supernatants were removed and analysed for the levels of IL-6 and IL-8 using ELISA.

5.3.5 Analysis of IL-6 and IL-8 in epithelial cell supernatants

ELISA kits specific for the measurement of human IL-6 and IL-8 (pre-coated with capture antibody) were prepared as per manufacturer's recommendations. Briefly, a known volume of standards and culture supernatants (100 µL and 50 µL for IL-6 and IL-8, respectively) was added to the pre-coated wells. Further, 50 µL/well of biotinylated detection antibody was added to each plate and incubated for 2 hours at room temperature and washed 5 times. Next, 100 µL/well of standard horseradish peroxidase conjugated streptavidin (a commonly used enzyme to modify substrate resulting in colour development) was added to each well and incubated for 30 minutes at room temperature in the dark. Plates were again washed 5 times and 100 µL/well of stabilised chromogen was added to each well. Plates were then allowed to stand in the dark for 30 minutes and the reaction was quenched using 100 µL/well of stop solution (1N hydrochloric acid). Measurement of the optical density was performed using a plate reader (Spectra Max M2 microplate reader, Sunnyvale, CA) at 450 nm. Each epithelial cell treatment was performed in triplicate and supernatants of each treatment were analysed in duplicate.

5.3.6 Viability and cytotoxicity studies of enoxaparin on epithelial cells

The effect of enoxaparin at maximal inhibitory concentration on either cellular viability of A549 cells or its cytotoxic effect on cells after 24 hours of incubation was assessed using two routinely used methods. The viability of cells was determined by the trypan blue exclusion. The cytotoxicity of enoxaparin treatment was determined using the LDH in-vitro toxicology assay, as described before [149]. Briefly, the culture supernatants were centrifuged at 250g for 4 minutes. An aliquot of either blank (complete medium) or control (epithelial cells only) cells treated with trypsin or

co-incubated with enoxaparin, was mixed with an aliquot of solution containing LDH assay mixture (LDH substrate, LDH dye and LDH cofactor). The mixture was incubated at room temperature for 20-30 minutes and the reaction was quenched by the addition of 1 N hydrochloric acid (15 µL). The absorbance was measured spectrophotometrically using a plate reader (Spectra Max M2 microplate reader, Sunnyvale, CA) at a wavelength of 490 nm. Each sample was prepared and analysed in triplicate.

5.3.7 Separation of enoxaparin

The chromatographic separation of enoxaparin was carried out using a previously described ion-exchange chromatography (IC) technique [105] with minor modifications. Separations were performed on a biocompatible chromatography system (Thermo Fisher Scientific, NSW, Australia) using a semi-preparative Dionex CarboPac PA100 (250 mm, 9 mm ID, 8.5 µm) strong anion-exchange column (Thermo Fisher Scientific, NSW, Australia). The chromatography system consisted of a HPG-3400RS binary separation pump, WPD-3000RS auto-sampler, TCC-3000RS column thermal compartment and UV-3000RS detector. The column thermal compartment was set at a temperature of 40°C and UV detection was performed at 232 nm. An injection volume of 250 µL was chosen for this work, which allowed 10 mg of enoxaparin to be loaded onto the column for each injection, and a total flow rate of 2.0 mL/minute was maintained throughout. Instrument control and data acquisition was performed using Chromeleon® software. The mobile phases were composed of Milli-Q water (A) and 2 M NaCl (B). The 2 M NaCl stock solution was prepared by mixing NaCl and Milli-Q water and the solution was filtered and degassed off-line. The optimised NaCl eluent gradient (mobile phase B) was from 32

to 74% over 0 to 70 minutes. The 14 fractions of enoxaparin were collected in centrifuge tubes over the elution period between 29 to 65 minutes. The collected fractions containing NaCl (0.64-1.48 M) were then subjected to desalting procedures.

5.3.8 Desalting of enoxaparin fractions

The IC-derived fractions of enoxaparin were desalted as described previously [105] with minor modifications. Each fraction was concentrated on a miVac DNA centrifugal concentrator (Genevac Ltd, Suffolk, UK) at 40°C. The concentrated solutions containing NaCl and enoxaparin fractions were desalted using PD MidiTrap G-10 columns (GE Healthcare Life Sciences, Uppsala, Sweden) having desalting capacity of more than 95%. The recovery of each fraction was determined by reanalysing the desalted fractions using IC under the same chromatographic conditions as described above. The concentration of each fraction was calculated using the differences in the peak areas of the desalted fraction and enoxaparin fraction eluted at the same time.

The stock solution (1 mg/mL) of each fraction was prepared in serum-free Ham's F12K medium. The fractions were tested for their effects on the release of IL-6 and IL-8 from stimulated human pulmonary epithelial cells, as described above. Anticoagulant activity of the fraction responsible for the inhibitory effect of enoxaparin was determined using an anti-factor Xa assay. Its structural characterisation was then carried out using a nuclear magnetic resonance (NMR) technique.

5.3.9 NMR analysis of enoxaparin fraction

The identified fraction was dissolved in potassium phosphate buffer at pH 7.0 prepared in 99.9% D_2O. All data were acquired with a Bruker AVANCE III HD 4-channel spectrometer with a 5mm triple resonance cryogenically cooled probe. Sample temperature was regulated at 298K and spectrometer field frequency was 600.07 MHz and 150.88 MHz for ^{1}H and ^{13}C nuclei, respectively. Data acquisition and processing were executed within Topspin™ 3.2 software (Bruker Corporation, MA, USA). All chemical shifts are referenced indirectly to DSS (NMR standard) at 0.0 ppm in ^{1}H and ^{13}C. The experiments conducted and their relevant acquisition and processing details are as follows: ^{1}H-1D ("noesgppr1d") with presaturation of residual solvent resonance, acquired with 64K datapoints in 8 transients, spectral width of 10 ppm and a relaxation delay of 4 seconds. Raw data was processed with an exponential apodisation with a line-broadening factor of 0.3 Hz prior to Fourier transformation; ^{13}C-1D ("zgpg30") with proton decoupling acquired with 64K datapoints in 20,000 transients, spectral width of 200 ppm and relaxation delay of 2 seconds. Raw data was processed with an exponential apodisation with a line-broadening factor of 1.0 Hz prior to Fourier transformation; ^{1}H-^{13}C-HSQC ("hsqcedetgpsisp2.2") with multiplicity editing acquired with 2048 × 64 datapoints, 2 transients per 2D increment, spectral widths of 10 ppm and 165 ppm for ^{1}H and ^{13}C dimensions, respectively, and a relaxation delay of 1.5 seconds. Data were processed with zero filling in both dimensions and a squared sine apodisation (SSB=2) to a resultant matrix of 4096 × 1024 datapoints; ^{1}H-^{13}C-HMBC ("hmbcgpl2ndqf") acquired with 2048 × 128 datapoints, 16 transients per 2D increment, spectral widths of 10 ppm and 200 ppm for ^{1}H and ^{13}C dimensions, respectively, and a relaxation delay of 1.5 seconds. Data were processed with zero filling in both dimensions and a squared sine apodisation

(SSB=2) to a resultant matrix of 4096 × 1024 datapoints; ^{1}H-^{13}C-HSQCTOCSY ("hsqcetgpml") acquired with 2048 × 256 datapoints, 32 transients per 2D increment, spectral widths of 10 ppm and 165 ppm for ^{1}H and ^{13}C dimensions, respectively, TOCSY mixing time of 120 milliseconds and a relaxation delay of 1.5 seconds. Data were processed with zero filling in both dimensions and a squared sine apodisation (SSB=2) to a resultant matrix of 4096 × 1024 datapoints.

5.3.10 Desulfation of enoxaparin fraction

5.3.10.1 *Selective 2-O desulfation*

Selective 2-*O*-desulfation of identified fraction was performed using a previously described method [151]. Briefly, a solution containing 8 mg/mL of fraction was dissolved in 200 µL of 0.1 M sodium hydroxide. The mixture was then frozen and lyophilised. The residues were dissolved in 0.5 mL of Milli-Q water and acetic acid was added to adjust the pH of the mixture to 8.0. The mixture was evaporated to dryness and precipitated using 80% *v/v* of anhydrous methanol, followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was carefully discarded and samples were kept at 4°C overnight. Any traces of methanol were removed using a miVac DNA centrifugal concentrator and the remaining precipitants were dissolved in 4 mL Milli-Q water to obtain 2-*O*-desulfated fraction of enoxaparin.

5.3.10.2 *N-desulfation*

A solution containing 8 mg/mL of enoxaparin fraction was mixed with 650 µL of tetrahydrofuran and 50 µL of Milli-Q water and incubated for 30 minutes at 50°C for partial *N*-desulfation, as described previously [151] with minor modifications. The solution was neutralised using 0.1 M sodium hydroxide. The resulting mixture was

evaporated to dryness and precipitated by the addition of anhydrous methanol as described above and dissolved in 4 mL Milli-Q water.

5.3.10.3 *Selective 6-O-desulfation*

Selective 6-*O*-desulfation of enoxaparin fraction was performed as previously described [151]. Briefly, 8 mg/mL of the fraction was mixed with 1 mL of solvent (tetrahydrofuran) and 1 mL of silylating agent (*N*-methyl-*N*-(trimethylsilyl) trifluroacetamide). The solution was incubated for 9 hours at 50°C. The resulting mixture was evaporated to dryness and precipitated by the addition of anhydrous methanol as described above and the precipitants were dissolved in 4 mL Milli-Q water.

After desulfation, free sulfate groups were removed using a 600Da cut-off filter (Millipore, NSW, Australia) at 15000 rpm for 10 minutes. The concentrated supernatant was dissolved in 1 mL of Milli-Q water for further use. Each selectively desulfated enoxaparin sample was tested for its effect on the release of IL-6 and IL-8 from stimulated human pulmonary epithelial cells. Each sample was prepared and analysed in triplicate.

5.3.11 Binding of proteins to the identified fraction

5.3.11.1 *High-performance size-exclusion chromatography (HP-SEC) instrumentation*

The putative binding of the identified fraction to either trypsin or ILs was determined by HP-SEC. The HP-SEC system (Thermo Fisher Scientific, NSW, Australia) consisted of a HPG-3400RS binary separation pump and a WPS-3000TRS auto-sampler. The system was connected to Corona Ultra RS charged aerosol detector (C-

CAD; Thermo Fisher Scientific, NSW, Australia). C-CAD was used as per the manufacturer's recommended settings of 35±0.2 psi for the nitrogen gas flow at 30°C nebuliser temperature. An injection volume of 10 µL and a total flow rate of 1.0 mL/minute were maintained. Instrument control and data acquisition were performed using Chromeleon® software.

5.3.11.2 *HP-SEC analysis*

HP-SEC analysis was performed using two HP-SEC columns coupled in series; a Superdex™ peptide 10/300 GL (300 × 10 mm) and a Superdex™ 75 10/300 GL (300 × 10 mm) column (GE Healthcare Life Sciences, Uppsala, Sweden) were used for this purpose. Isocratic elution of the tested analytes was performed with a mobile phase containing 100 mM ammonium acetate (pH 6.0) as eluent. The method was validated by investigating the intra- (n=6) and inter-day (five consecutive days; n=30) precision using peak areas of 10 µM of UFH, thrombin, trypsin, IL-6 or IL-8 or 100 µM of identified fraction. Mean intra- and inter-day accuracy was also determined and calculated as (observed concentration-expected concentration)/expected concentration × 100). Mean inter-day peak retention time for each of the analytes was also determined. The peak area and retention time of the analytes were determined using Cobra™ integration wizard software.

5.3.11.3 *Sample preparation for HP-SEC analysis*

A stock solution containing 20µM of UFH, thrombin, trypsin, IL-6 or IL-8 or 1000 µM of identified fraction was prepared in potassium phosphate buffer. The stock solutions of thrombin, trypsin, IL-6 or IL-8 was diluted in ammonium acetate to obtain the concentration of 10 µM. The stock solution of UFH or identified fraction

was diluted in Milli-Q water to obtain the concentration of 10 or 500 µM. Each sample was prepared and analysed in triplicate.

5.3.12 Statistical analysis

Data are presented as mean ± standard deviation (SD) or as percentage change in the release of IL-6 and IL-8 following different types of treatments compared to trypsin-stimulated controls. Statistical analysis was performed using GraphPad Prism (version 6, GraphPad Software Inc, CA, USA), and significance was evaluated using independent sample or paired Student's *t*-test, and one way analysis of variance (ANOVA), where applicable, followed by Dunnett's multiple comparison test. A *p*-value of <0.05 was considered statistically significant.

5.4 RESULTS AND DISCUSSION

5.4.1 Release of IL-6 and IL-8

The calibration curves used for the measurement of IL-6 and IL-8 were generated using seven recommended concentrations of respective IL standards. The linearity, estimated by correlation coefficient (r^2), was greater than 0.988. The levels (pg/mL) of IL-6 and IL-8 measured 24 hours after trypsin-induced stimulation of A549 epithelial cells are shown in Figure 5.1. The baseline levels of IL-6 and IL-8 were 42 and 26 pg/mL, respectively. In the presence of trypsin, the levels of IL-6 and IL-8 were increased to 800 pg/mL and 2900 pg/mL ($p<0.0001$). In the current study, trypsin was used to activate the lung epithelial cells because it can induce the stimulation of proteinase-activated receptors (PAR) expressed by human alveolar as well as bronchial epithelial cell lines [232]. Recent *in-vivo* studies have shown that endogenous trypsin (mainly located in the lung) induced activation of PAR in human

lung epithelial cells releases IL-6 and IL-8, resulting in the progressive loss of lung functions [233].

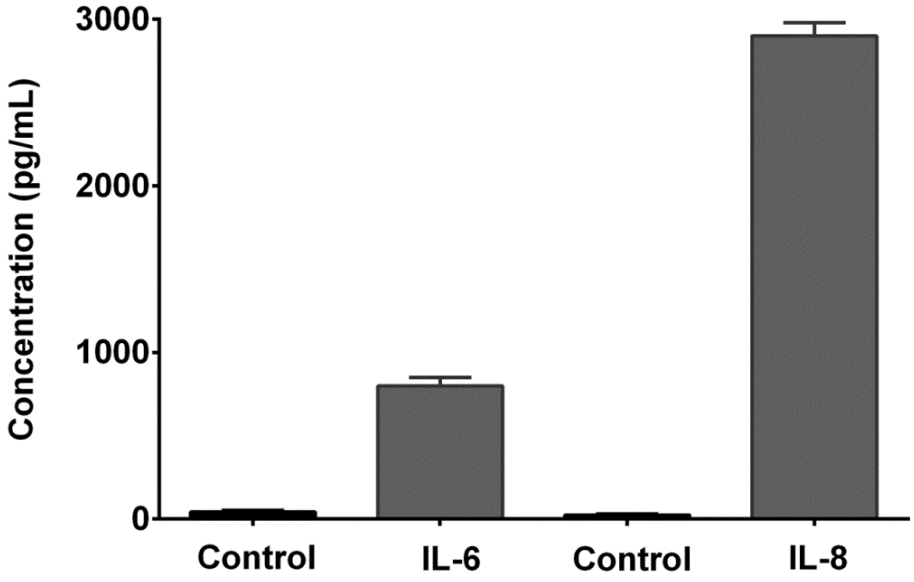

Figure 5.1 *In-vitro* interleukin release. Trypsin-induced release of IL-6 and IL-8 from the epithelial cell culture supernatants. Data is presented as mean ± SD.

5.4.2 Effect of enoxaparin on IL-6 and IL-8 release

As IL-6 and IL-8 are likely to be a potential target for new treatment modalities for this important disease, their release from the lung epithelial cells was determined in the presence or absence of enoxaparin. It inhibited the release of IL-6 and IL-8 in a concentration dependent manner (Figure 5.2A and 5.2B). Compared to control samples, the levels of tested ILs were not statistically different in the presence of 5 and 10 µg/mL of enoxaparin (p>0.1466). The maximum inhibition was observed at 60 µg/mL where the release of IL-6 and IL-8 was inhibited by 31% and 37%, respectively (p<0.0001). A further increase in the concentration of intact enoxaparin did not result in greater inhibition of IL release. LMWHs have been reported to inhibit the release of various inflammatory cytokines, including IL-4, IL-5, IL-13 and tumour

necrosis factor-alpha [145, 190, 234]. However, the current study for the first time demonstrated that enoxaparin can significantly inhibit the release of IL-6 and IL-8 from the stimulated lung epithelial cells.

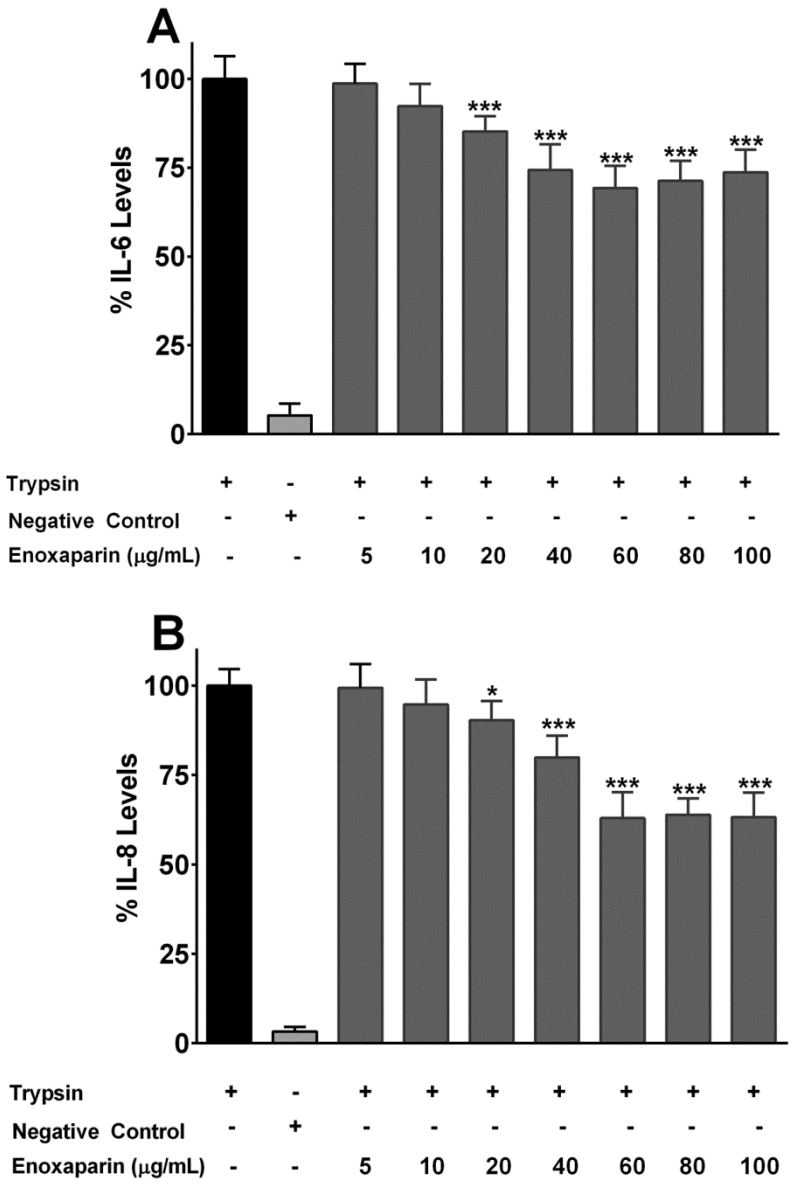

Figure 5.2 Concentration-dependent effect of enoxaparin on interleukin release. Effect of different concentration of enoxaparin on the release of IL-6 **(A)** and IL-8 **(B)** following trypsin-induced *in-vitro* stimulation epithelial cells. Data is presented as percentage (mean ± SD) of the maximal observed IL-6 and IL-8 concentrations. *p<0.05, and ***p<0.001 versus trypsin-stimulated control.

5.4.3 Effect of enoxaparin on cell viability and proliferation

To rule out that the observed inhibitory effect was because of enoxaparin-induced cytotoxicity or inhibition of cell proliferation, the cell viability and proliferation assays were performed in the presence or absence of intact enoxaparin. The concentration of 60 μg/mL of intact enoxaparin was selected to determine its effect on the viability and proliferation of A549 human pulmonary epithelial cells. After 24 hours of incubation, 60 μg/mL of enoxaparin did not reduce the viability of epithelial cells compared to the control. Similarly, no effect on the number of cells (proliferation) was observed after the addition of 60 μg/mL of enoxaparin compared to the control (Figure 5.3A and 5.3B). Additionally, enoxaparin did not induce cellular toxicity since it was not found to increase the release of LDH in epithelial cell culture supernatants (Figure 5.3C). These results indicated that the suppression of IL-6 or IL-8 release by enoxaparin was not related to its cytotoxic effect or changes in cellular proliferation.

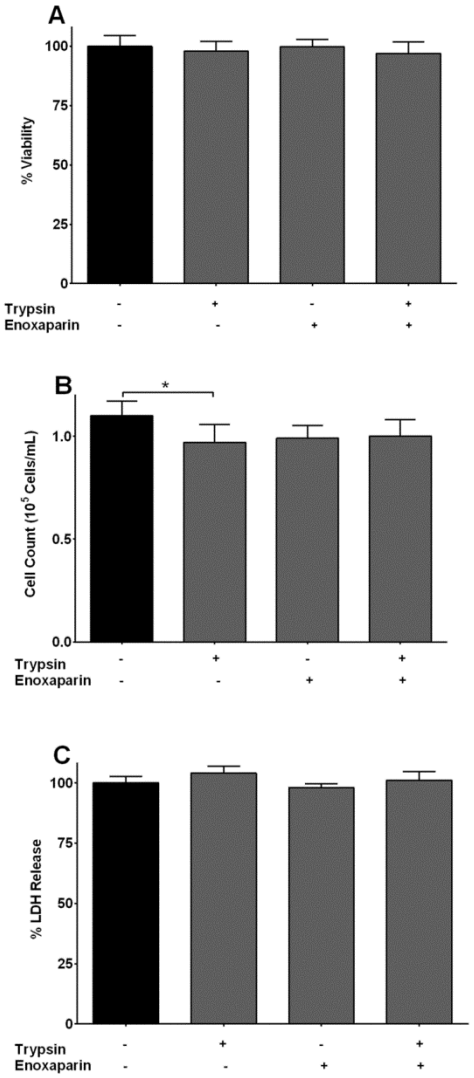

Figure 5.3 Effect of enoxaparin on cellular viability and proliferation. (A) Viability of epithelial cells was determined by trypan blue dye exclusion test and is presented as % of viable cells remaining after 24 hours of incubation with trypsin, enoxaparin or trypsin + enoxaparin. Data is presented as mean ± SD. **(B)** Proliferation of epithelial cells was determined after 24 hours in the presence of trypsin, enoxaparin or trypsin + enoxaparin. Cells were counted after incubation for 24 hours and expressed as 10^5 cells/mL. Data is presented as mean ± SD. *p<0.05 versus unstimulated control. **(C)** Viability of epithelial cells is presented as mean % LDH release ± SD. Cells were incubated for 24 hours in the presence of trypsin, enoxaparin or trypsin + enoxaparin.

5.4.4 Effect of enoxaparin fractions on IL-6 and IL-8 release

Enoxaparin is a mixture of structurally complicated and unidentified fractions. It has been suggested that different fractions of enoxaparin have different biological activities. For example, the pentasaccharide sequence (5 saccharides) of enoxaparin is required for its anticoagulant effect [235]. On the other hand, hexasaccharides (6 saccharides) of enoxaparin are effective in inhibiting the macrophage-induced release of nitric oxide [105]. Therefore, to find out the fraction(s) responsible for the observed inhibitory effect of the parent compound, enoxaparin was separated into different fractions using an IC technique (Figure 5.4A). The effects of 14 IC-derived fractions on the release of IL-6 and IL-8 are shown in Figure 5.4B and 5.4C. The 14 fractions were selected based on their previously reported molecular weights [105]. For example, the molecular weight of fraction 1, fractions 2, 3 and 4, and fraction 5, 6 and 7 were found to be approximately 600, 1200 and 1800 Da, respectively. The tested concentration of each fraction reflected their concentration present in 60 μg/mL of enoxaparin (at which enoxaparin exhibited the maximal level of IL-6 and IL-8 inhibition).

Various fractions inhibited the release of IL-6 and IL-8 to different extents. While the majority of fractions did not significantly change the levels of IL-6 and IL-8 ($p>0.0614$), fraction 1 inhibited the release of IL-6 and IL-8 release by 30% and 35%, respectively ($p<0.0001$), and this effect was comparable to the suppression displayed by enoxaparin. Fraction 2 and 4 were found to inhibit the release of IL-6 and IL-8 by 10 and 12%, respectively ($p=0.0274$ and 0.0230). Since fraction 1 of enoxaparin showed the maximum inhibition of tested ILs, it was selected for further analytical and bio-analytical investigations.

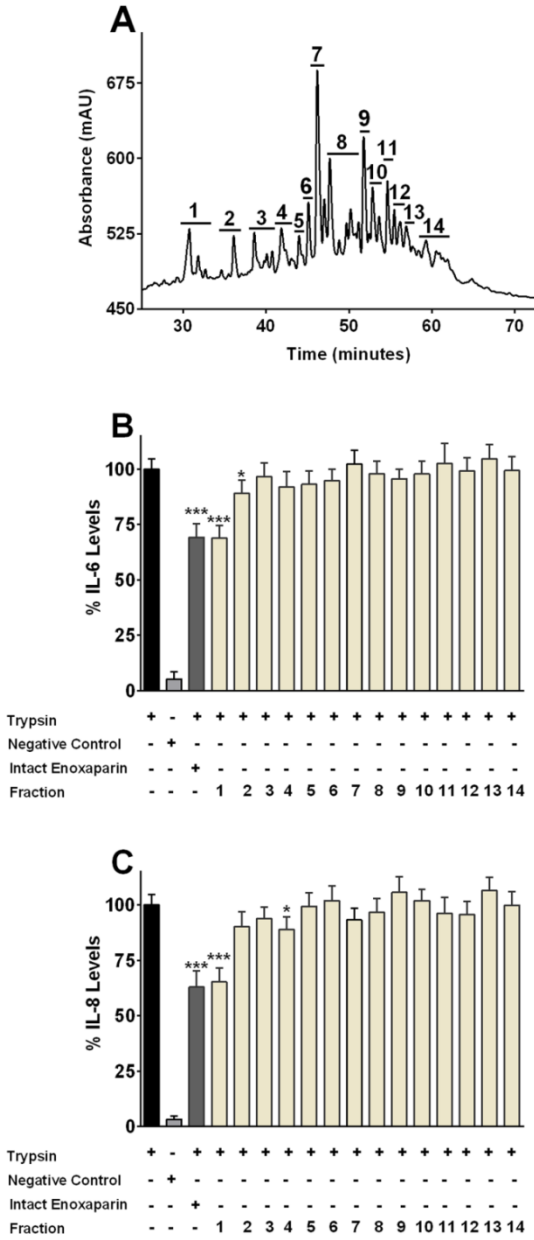

Figure 5.4 Effect of enoxaparin fractions on interleukin release. Ion-exchange chromatographic (IC) separation of enoxaparin. The separations were performed using a CarboPac PA100 semi-preparative column; optimised NaCl gradient was from 32-74% over 70 minutes with a flow rate of 2 mL/minute and UV detection wavelength of 232 nm. The numbers indicate the area of all the fractions collected. Data represents a typical experiment out of ten independent experiments **(A)**. Inhibition of IL-6 **(B)** and IL-8 **(C)** release by IC-derived 14 fractions of enoxaparin (60 µg/mL) after trypsin-induced *in-vitro* stimulation of epithelial cells. The relative percentile amount of each fraction (fraction 1 to 14) present in 60 µg/mL of intact enoxaparin was: 9.18%, 4.24%, 6.12%, 5.15%, 2.99%, 4.78%, 20.32%, 11.4%, 10.53%, 7.35%, 5.98%, 4.6%, 2.95% and 5.06% respectively. Data is presented as percentage (mean ± SD) of the maximal observed IL-6 and IL-8 concentrations. **p*<0.05, and ****p*<0.001 versus trypsin-stimulated control.

5.4.5 Concentration-dependent effect of fraction 1 on IL-6 and IL-8 release

Like enoxaparin, fraction 1 also inhibited the release of IL-6 and IL-8 in a concentration-dependent manner (Figure 5.5A). The maximum inhibition of IL-6 and IL-8 was observed at 15 μg/mL and higher concentrations did not result in greater inhibition. At 15 μg/mL, it inhibited the release of IL-6 and IL-8 by 70 and 76%, respectively (p<0.0001).

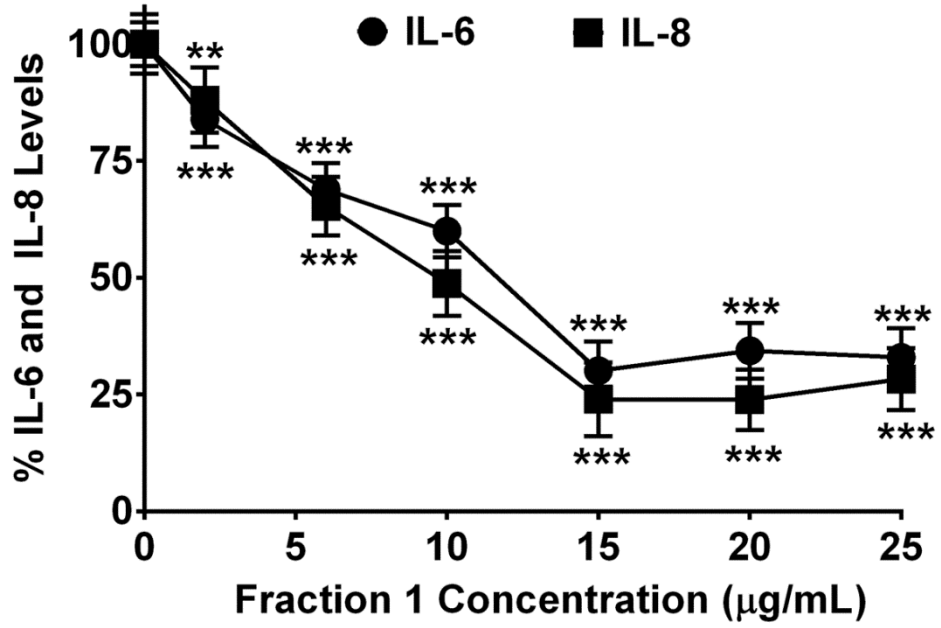

Figure 5.5 Concentration-dependent effect of IC-derived fraction 1 on interleukin release. Inhibition of IL-6 and IL-8 release by fraction 1 of enoxaparin (0 to 25 μg/mL) after *in-vitro* stimulation of epithelial cells via trypsin. Data is presented as percentage of trypsin-stimulated control (mean ± SD). **p<0.01 and ***p<0.001 versus trypsin-stimulated control.

Fraction 1 of enoxaparin, as can be seen in Figure 5.4A, is composed of three different sub-fractions eluting at 30.0, 31.2 and 32.3 minutes, respectively. Although the molecular weights of the three sub-fractions (fraction 1A, 1B and 1C) were found to be similar, the IC technique was efficient enough to separate them from each other. The separation of IC is based on the interaction of the negatively charged groups (e.g.

sulfate groups) of enoxaparin fractions with the positively charged stationary phase of the separation column. The fractions with more negative charge elute later during the IC salt gradient separation. Therefore, sub-fraction 1A appears to have less negatively charged groups than fraction 1B or fraction 1C. As the potential anti-inflammatory effects of LMWHs depend on their degree, as well as pattern, of sulfation, we further investigated the effect of each sub-fraction on the release of IL-6 and IL-8. The effect of three different sub-fractions on the release of IL-6 and IL-8 is shown in Figure 5.6. Sub-fraction 1A inhibited the release of IL-6 and IL-8 by approximately 65% and 74%, respectively ($p<0.0001$). On the other hand, sub-fraction 1B and 1C did not significantly inhibit the release of tested ILs ($p>0.1683$). As sub-fraction 1A was found to be responsible for the majority of the inhibitory effect of fraction 1, its structure was characterised by NMR.

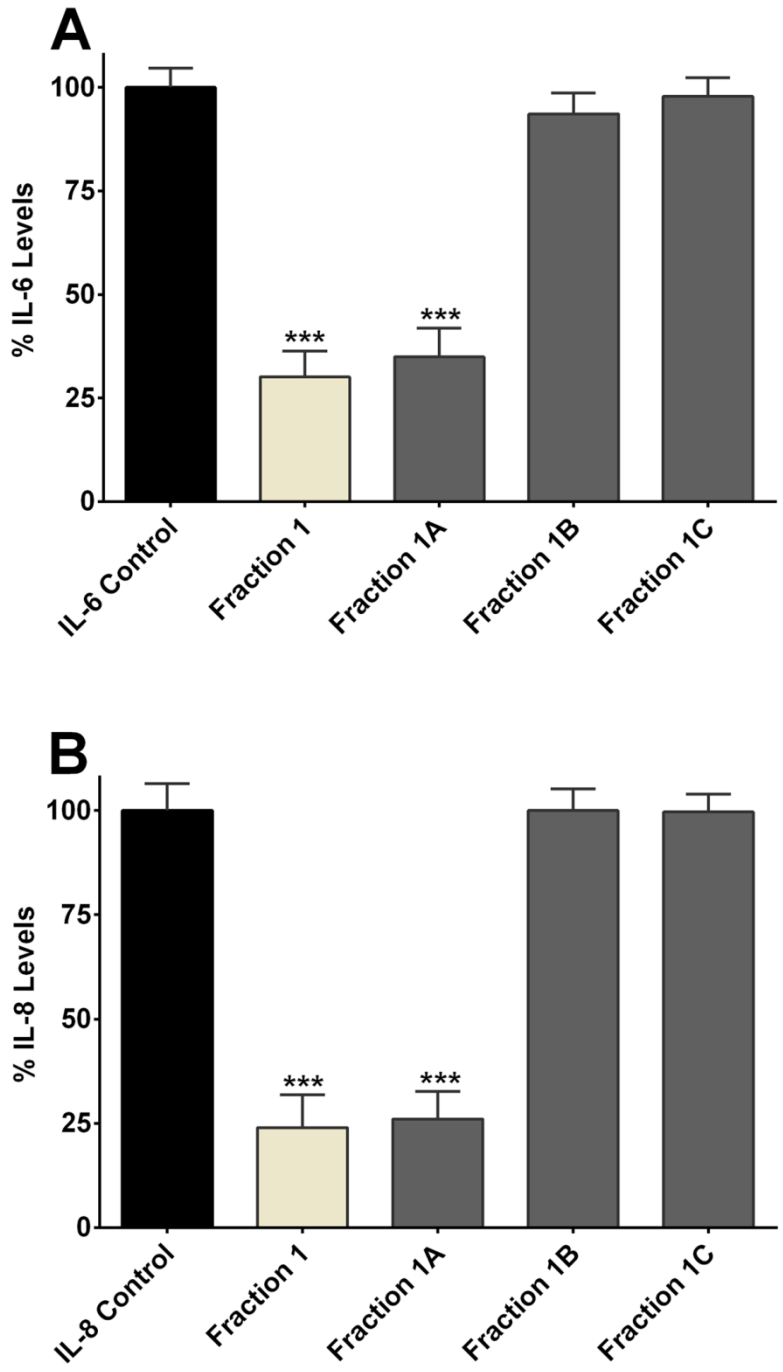

Figure 5.6 Effect of sub-fractions of IC-derived fraction 1 on interleukin release. Inhibition of IL-6 **(A)** and IL-8 **(B)** release by enoxaparin fraction 1 and its three different sub-fractions (1A, 1B and 1C) after trypsin-induced *in-vitro* stimulation of epithelial cells. Data is presented as percentage (mean ± SD) of the maximal observed IL-6 and IL-8 concentrations. ****p*<0.001 versus trypsin-stimulated control.

5.4.6 Structural characterisation of sub-fraction 1A

Full assignment of the dominant proton and carbon signals in the enoxaparin sub-fraction 1A was made from analysis of the NMR data. Proton connectivity networks in the individual sugar rings were established from correlations in ^{1}H-^{1}H TOCSY spectra. Carbon assignments were derived through one-bond correlation of these protons to carbons in the ^{13}C-^{1}H HSQC spectrum (Figure 5.7). The ^{13}C-^{1}H HSQC-TOCSY experiment (Figure 5.8) was used to discriminate between groups of interconnected protons in separate sugars for which overlap in the ^{1}H-^{1}H-TOCSY did not allow unambiguous assignment. The link between the two sugars was confirmed by a correlation across the glycosidic linkage between H4' and C1 observed in heteronuclear multiple-bond correlation (HMBC) spectrum. The NMR assignments and measured J-couplings of sub-fraction 1A are summarised in Table 5.1, with atom numbering consistent with previously reported data [175].

Table 5.1 **NMR assignments of enoxaparin disaccharide**

	1	**2**	**3**	**4**	**1'**	**2'**	**3'**	**4'**	**5'**	**6'**
^{1}H ppm	5.435	4.496	4.263	5.89	5.379	3.192	3.673	3.754	4.084	4.141 4.285
$^{3}J_{HH}$ Hz	3.36	3	3	4.44	3.48	10.16 3.57	10.43	9.39	9.98 3.57	11.26 3.8
^{13}C ppm	96.61	75.05	63.41	106.3	90.94	57.71	68.94	78.39	67.99	66.7

Atoms in the glucosamine unit are denoted by the prime superscript
Cq (COO-) 169.1 ppm, C6 (Ido) 144.79 ppm

It was found to be a disaccharide of enoxaparin containing a unit of α-L iduronic acid and one of α-D glucosamine-6-sulfate. The glucosamine unit of the disaccharide was present in both α and β forms, and could be correlated with chemical shift and coupling observations for a similar disaccharide fragment of heparin [175] and spectra for glucosamine-6-sulfate in the Human Metabolome Database (HMDB) repository [236]. The iduronic acid unit did not display evidence of multiple conformations at the temperature used in this study. The glucosamine was predominantly in the α-form based on relative integral volumes between the two forms measured in the ^{1}H-1D spectrum.

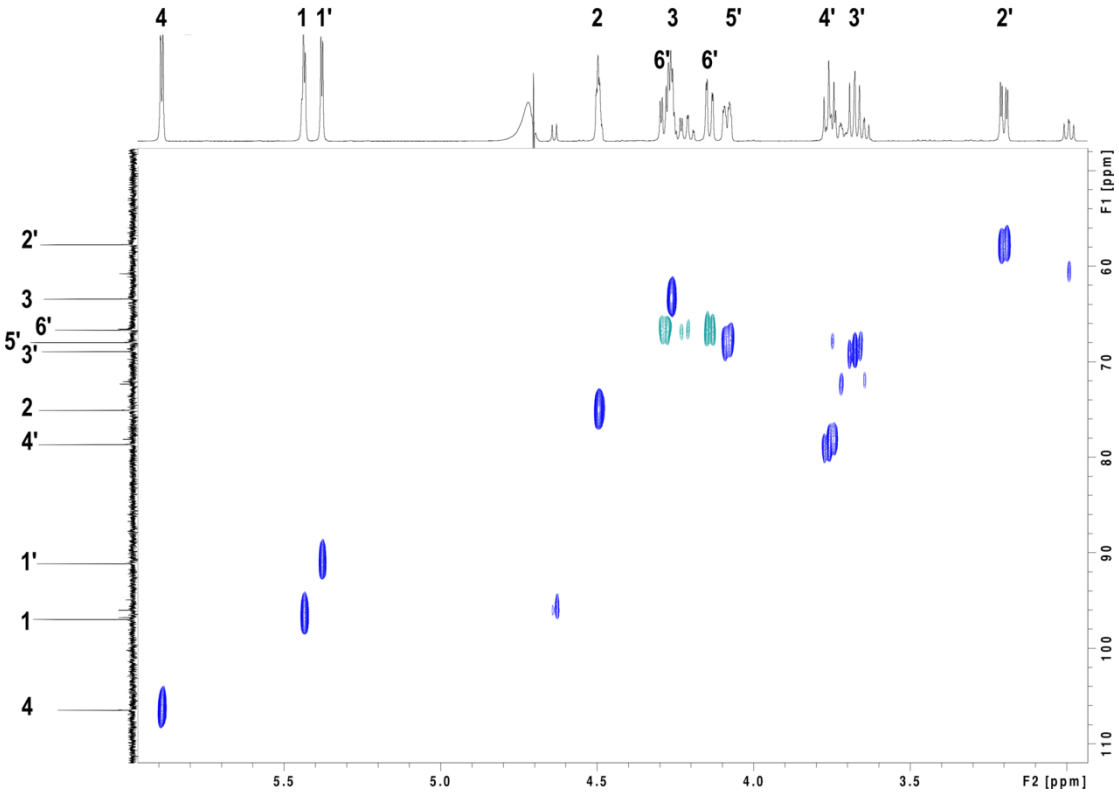

Figure 5.7 ^{13}C-^{1}H HSQC multiplicity edited spectrum for enoxaparin sub-fraction 1A. Axis units are chemical shifts in parts per million (ppm). The dark blue contours denote correlations between carbon atoms with one or three attached protons and cyan contours carbons with a single attached proton. The atom nomenclature from Table 1. is applied to the proton and carbon axis projections.

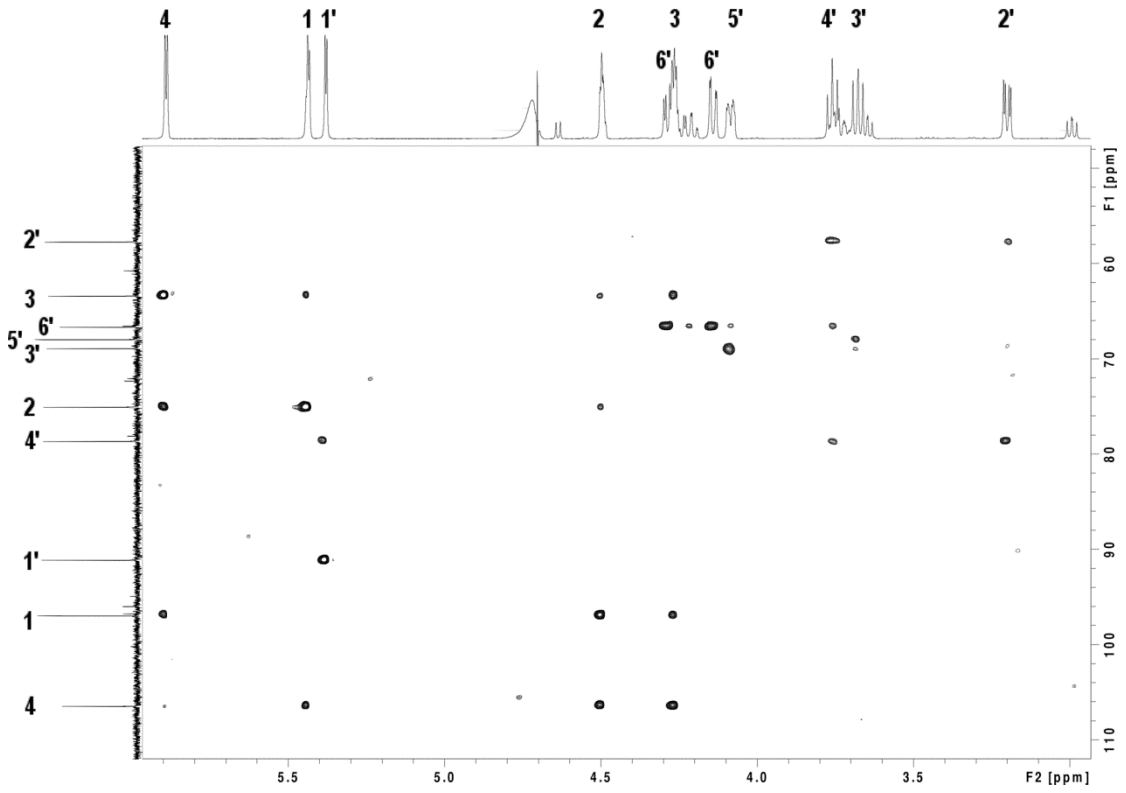

Figure 5.8 ^{13}C-^{1}H HSQC-TOCSY 120ms spectrum for enoxaparin sub-fraction 1A. Axis units are chemical shifts in parts per million (ppm). The atom nomenclature from Table 1. is applied to the proton and carbon axis projections.

5.4.7 Anti-factor Xa analysis of disaccharide of enoxaparin

The anti-factor Xa activity of fourteen IC-derived fractions of enoxaparin is shown in Figure 5.9. As expected, the disaccharide of enoxaparin did not show any anti-factor Xa activity. Inhibition of factor Xa by anti-thrombin requires a formation of a complex composed of factor Xa, anti-thrombin and enoxaparin [237]. To mediate an interaction between factor Xa and anti-thrombin, a minimum chain length of 5 saccharide (pentasaccharide) sequence of enoxaparin is required. It means the pentasaccharide of enoxaparin is the smallest fraction required to mediate inactivation of factor Xa by anti-thrombin. The identified disaccharide of enoxaparin was too small to act as a template through which anti-thrombin mediated inactivation of factor

Xa can occur. This finding is significant because the risk of bleeding is increased when enoxaparin is used for medical conditions other than where an anticoagulation effect is required. The identified disaccharide lacked the anticoagulant activity and therefore can eliminate the potential risk of bleeding associated with enoxaparin.

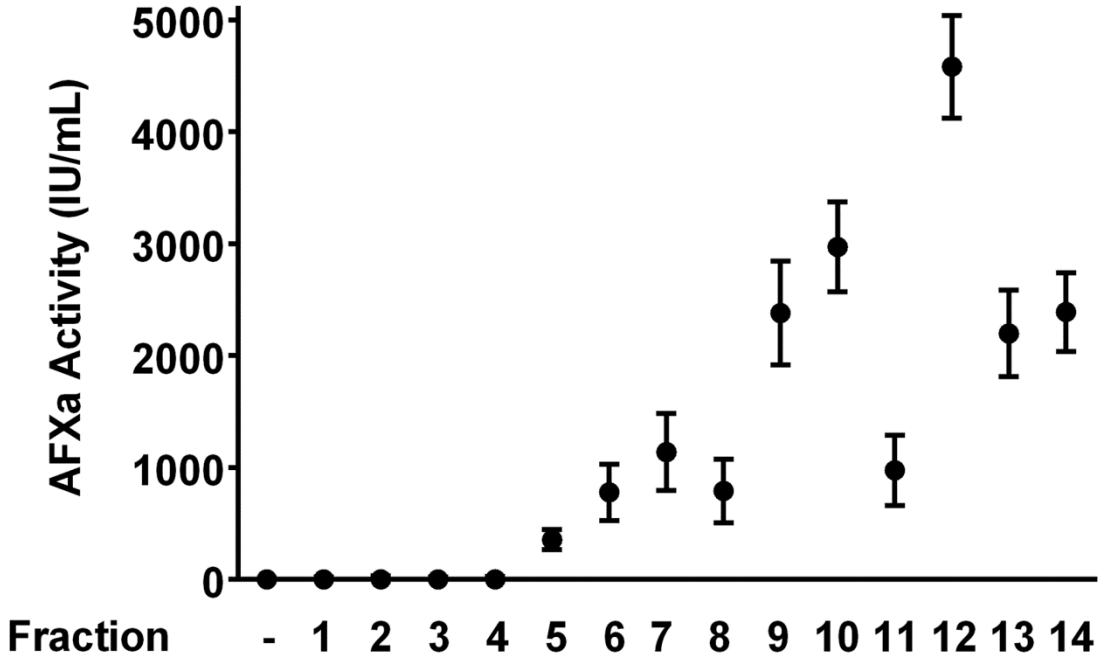

Figure 5.9 Anti-factor Xa activity of fourteen enoxaparin fractions derived using IC technique. The anti-factor Xa assay is described in the experimental section. Data is presented as mean ± SD (n=3).

5.4.8 Effect of desulfated disaccharide of enoxaparin on IL-6 release

The presence of sulfate groups at specific positions, such as 2-*O*, *N*- or 6-*O*, are known to be essential to elicit an anti-inflammatory response to heparins [231]. Therefore, the selectively desulfated disaccharide was tested for its ability to inhibit the release of IL-6 (Figure 5.10). As expected, the disaccharide inhibited the release of IL-6 by approximately 70% (p<0.0001). While 2-*O* and *N*-desulfated disaccharide did not significantly change the observed inhibition of disaccharide (p>0.6018), the disaccharide-induced inhibition of IL-6 was reduced by 98% in the presence of 6-*O*-

desulphated disaccharide (p<0.0001). The importance of *O*-sulfate groups in controlling the anti-inflammatory activity of heparins has been reported before. For instance, the presence of sulfate groups at *O*-position was found to be critical for: i) binding to inflammatory cytokines and chemokines, such as IL-4 and CXCL-12 [215, 218], ii) formation of covalent complexes with proteolytic enzymes, such as thrombin [238] and iii) binding to various receptors, such as fibroblast growth factor receptors [239]. The present study, for the first time, reports the requirement of 6-*O* sulfate groups for the inhibition of IL-6 and IL-8 release from human pulmonary epithelial cells.

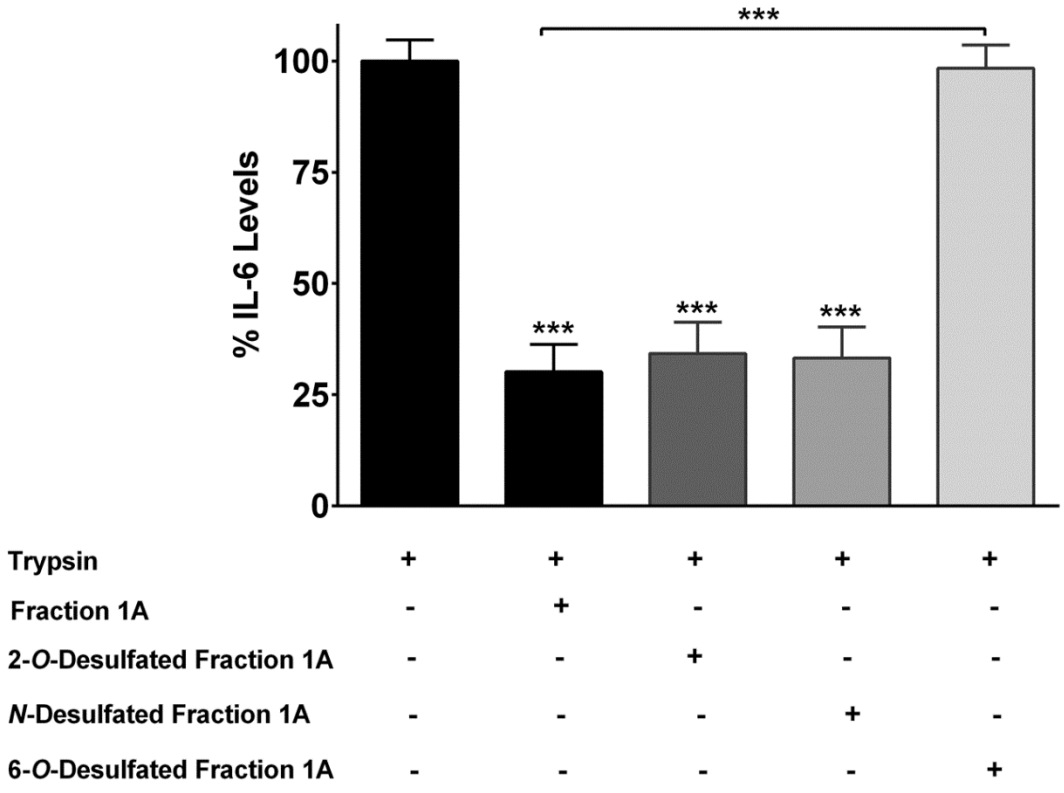

Figure 5.10 Effect of various desulfated fraction 1A on IL-6 release. Epithelial cells were stimulated with trypsin in the presence of either 2-*O*-desulfated, *N*-desulfated or 6-*O*-desulfated fraction 1A of enoxaparin. Data is presented as mean ± SD. ***p<0.001 versus trypsin-stimulated control.

5.4.9 Binding of proteins to disaccharide of enoxaparin

Decreased levels of IL-6 and IL-8 observed through ELISA could potentially be because of: i) direct binding of disaccharides to trypsin and, hence, inhibiting the trypsin-induced activation of PAR (lung epithelial cell receptors); ii) binding of disaccharides to IL-6 and IL-8 and, hence, their reduced detection through ELISA or iii) binding of disaccharides to PAR and, hence, inhibiting PAR-induced release of IL-6 and IL-8 through specific intracellular signalling pathways. Specific binding between glycominoglycans (e.g. enoxaparin) and proteolytic enzymes (e.g. thrombin) has been reported before [240]. Direct binding of glycosaminoglycan disaccharide and proteolytic trypsin could impair the ability of trypsin to bind and activate the PAR. Therefore, we needed to confirm that the observed inhibition of release of ILs was not due to this potential *in-vitro* artefact of our experimental cell system. Hence, HP-SEC was used to investigate the putative binding between disaccharide and trypsin. In the current HP-SEC method, two separation columns were connected in series. The first column (Superdex™ peptide 10/300 GL) allowed the separation of proteins and carbohydrates having low MWs (0.1 to 8 kDa) and the second column (Superdex™ 75 10/300 GL) provided the effective separation of analytes with high MWs (3 to 70 kDa). Moreover, the detection of the tested analytes was carried out using a charged aerosol detector (CAD) instead of ultra-violet (UV) detector. The advantage of CAD, as compared to UV detection, is that the peak areas of analytes are not dependent on a UV chromophore, for which extinction coefficients can vary by orders of magnitude depending on the chemistry of analyte, but are dependent on the mass of analytes. The performance of the HP-SEC method was determined using intra- and inter-day precision and accuracy, and the inter-day retention time of each analyte. The intra- and inter-day precision relative standard deviation (RSD) for each analyte peak was

less than 4.1% (n=6) and 5.3% (n=5), respectively. The intra- and inter-day accuracy RSD for each analyte peak was less than 4.3% and 5.9%, respectively. Inter-day retention time RSD for each of the analyte peaks were less than 0.7% (n=6).

The ability of HP-SEC to determine the binding between glycominoglycans and proteins was first investigated by analysing UFH (10 μM) and thrombin (10 μM). The HP-SEC chromatograms of UFH, thrombin and a mixture of UFH/thrombin (1:1 molar ratio) are shown in Figure 5.11A. UFH and thrombin peaks were eluted at 65 and 37 minutes. In HP-SEC analysis, the separation of analytes is based on their molecular size and, therefore, thrombin with a MW of 37 kDa eluted earlier than UFH (MW 15 kD). However, when a mixture containing UFH and thrombin was analysed (Figure 5.11A), a new peak was eluted at 16 minutes, suggesting the observed component had a higher MW compared to thrombin or UFH. When glycosaminoglycans bind to proteins, it results in the formation of a high MW complex. Therefore, the observed reduction in the peak areas of thrombin and UFH, and simultaneous elution of a new peak indicated the binding between UFH and thrombin. UFH is a mixture of complicated oligosaccharides ranging from disaccharides to oligosaccharides larger than 50-100 saccharides. Only oligosaccharides with a minimum of 18 saccharides can bind to thrombin and, therefore, as can be seen in Figure 5.11A, approximately 25% of UFH oligosaccharides did not bind to thrombin. The disaccharide of enoxaparin and trypsin was then analysed by HP-SEC. As shown in Figure 5.11B, trypsin and disaccharide were eluted at 53 and 136 minutes respectively. There was no difference in the chromatographic profile of disaccharide or trypsin when a mixture of trypsin/disaccharide (1:50 molar ratio) was injected into the HP-SEC system (Figure 5.11B). For example, the peak area of trypsin remained unchanged when a solution

containing either trypsin or mixture of trypsin and disaccharide was injected. Also, no extra peak was observed when a mixture was analysed suggesting the decreased levels of ILs were not because of the interaction between disaccharide and trypsin. Apart from proteolytic enzymes, glycosaminoglycans are shown to interact with cytokines, chemokines and growth factors. For example, heparins can bind to IL-10, MCP-1 and epidermal growth factors and then modulate their biological activity [241-243]. However the HP-SEC results indicated that the disaccharide of enoxaparin did not bind to either IL-6 or IL-8.

Trypsin is known to activate PAR by cleaving the receptor's extracellular N-terminal domain. This cleavage results in the formation of a new N-terminus which then interacts with trypsin causing activation of G-protein-coupled signal transduction pathway [244]. Interestingly, the N-terminal domain of various proteins is reported to be a binding site for heparins as well [245, 246]. For example, heparins bind to the N-terminal domain of serine protease inhibitor resulting in the anticoagulant effect of enoxaparin [247]. Since, disaccharide of enoxaparin did not interact with either trypsin or the tested ILs, the observed decrease in IL levels could potentially be due to the interaction between N-terminal domain of PAR and disaccharide. Future experiments should be aimed to provide direct evidences of interactions between disaccharide of enoxaparin and N-terminus of PAR as well as the mechanistic insights via which the potential interaction occurs. In the current study only a single type of epithelial cell line was tested to investigate the effect of enoxaparin on the release of IL-6 and IL-8. However, upon stimulation, other types of epithelial cell lines with potentially different types of cell surface receptors also release pro-inflammatory mediators. Therefore, it would be interesting to investigate the effects of enoxaparin on such types of epithelial cells.

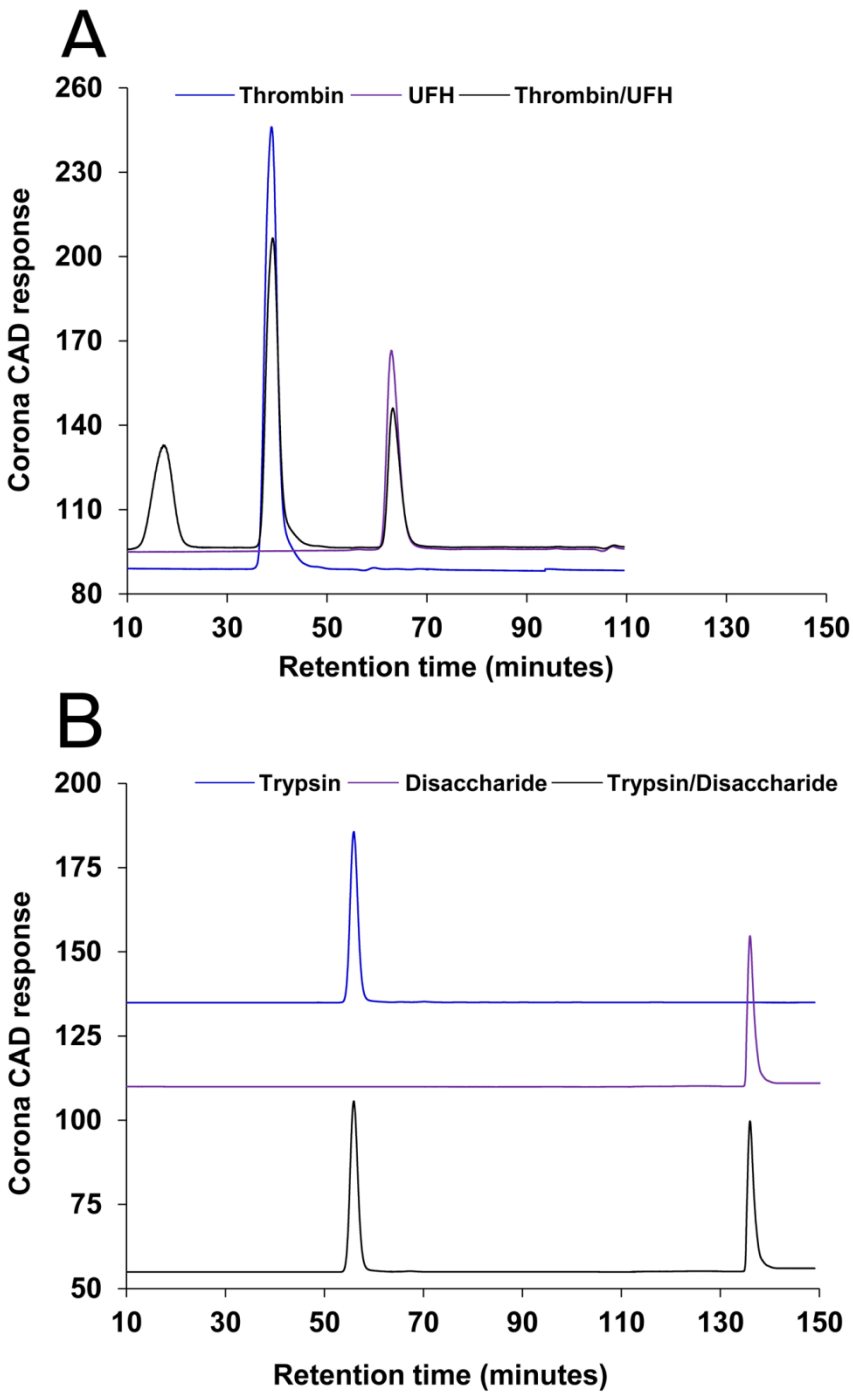

Figure 5.11 Binding of proteins with disaccharide of enoxaparin. Interaction of UFH with thrombin. HP-SEC chromatograms of UFH (10 μM), thrombin (10 μM) and a mixture of UFH/thrombin (1:1 molar ratio) **(A)**. Interaction of IC-derived disaccharide with trypsin. HP-SEC chromatograms of enoxaparin disaccharide (500 μM), trypsin (10 μM) and a mixture of trypsin/disaccharide (1:50 molar ratio) **(B)**. Details of the HP-SEC experimental conditions are given in Methods.

5.5 CONCLUSION

In summary, the identified fraction responsible for the anti-inflammatory effect of enoxaparin is composed of two saccharides. Therefore, it avoids the risk of bleeding as a minimum of pentasaccharide sequence is required for the anticoagulant effect. As IL-6 and IL-8 are important pro-inflammatory mediators involved in the early pathogenesis of asthma, inhibition of their release, as seen with the identified disaccharide, could provide a much needed novel therapeutic option for the management of clinical manifestations associated with this medical condition. However, future clinical studies should be conducted to confirm the preliminary findings of the current *in-vitro* study.

CHAPTER SIX

Challenges, Scientific Progress and Future Directions

Redefining Approaches to Asthma: Bridging the Gap between Heparin and Anti-inflammation

It will shortly be the 100th anniversary of the discovery of heparin; enormous progress has since been made in understanding the mechanistic insight of this complex biological as an anticoagulant and recently as an anti-inflammatory agent. Identification of the molecular basis via which heparin exert its characteristic anticoagulant effect represented a landmark in the research of this macromolecule. It was recognized that only a small structure within heparin also referred to as a pentasaccharide sequence was solely responsible for its active principle by eliciting a high inhibitory action against the final two proteases of the coagulation cascade.

Depolymerisation of unfractionated heparin generates heparin-derivatives also known as low-molecular-weight heparins (LMWHs). Heparins (unfractionated heparin and LMWHs) encompass a high degree of structural heterogeneity and complexity which make them one of the most information-dense molecules exhibiting multi-functional properties and hence, associated with a wide array of biological processes. Relatively recently, heparins have been shown to interact with manifold proteins and biological molecules, and thereby play a pivotal role in modulating inflammatory responses. As an anti-inflammatory agent, LMWHs like enoxaparin has been reported to possess clinical benefits in humans, including the once suffering from respiratory disorders like asthma, which is over and above its expected effects on blood coagulation. It seems arbitrary and still mysterious that a complex polysaccharide could be a useful anti-asthmatic agent.

Although, heparins remain one of the most widely used anticoagulant agent, their effect on blood coagulation is seen as an adverse effect when exploiting their

anti-asthmatic potential. Rapid developments of emerging new analytical tools and methodologies permitted the identification of heparin chains which were accountable for the features linked with its potent anti-asthmatic effects and were observed to be due to the presence of non-anticoagulant molecules in the parent compound which are entirely separable from its role as an anticoagulant. These advances in analytical capabilities represent a cornerstone allowing biological and pharmacological testing of the non-anticoagulant fragments in several inflammatory disorders including asthma. Numerous analytical techniques with their specific uses have been developed to separate anticoagulant and anti-inflammatory properties of heparins. For instance, molecular weight or sequence analysis of heparin fractions via gel-permeation chromatography or a much more challenging task of separating the whole polysaccharide using techniques like ion-exchange chromatography (IC). Several chemically or enzymatically modified analogues have also been tested for anti-inflammatory purposes. However, the anti-inflammatory potential of these analogues largely dependents on the efficacy of depolymerisation process since depolymerisation is known to produce structural modification in heparin fractions which may result in impaired the anti-inflammatory response. Questions still remain regarding their true pharmacologic potential, clinical limitations, safety and efficacy compared to the parent macromolecule.

Understanding the complex biology these molecules mediate, necessitates better understanding of their structural information. The anti-inflammatory properties of heparins are known to be sequence-dependent and varying sizes of non-anticoagulant sequences are reported to bind or inhibit individual proteins involved in the process of inflammation. Alongside the sequence dependency, conformationally defined patterns of sulphates were also shown to be critical and form the basis for

regulating the interaction with proteins and thereby induce biological activities. However, it has been recognised after selective desulfation of non-anticoagulant heparin fragments that some of the sulfate groups are compatible but are not essential for high affinity binding or inhibition of proteins. With a few exceptions, for the majority of anti-inflammatory effects, the knowledge of the active domains of non-anticoagulant heparin molecules is still incomplete. However, increasing interest in exploiting the pharmacology of non-anticoagulant heparin have led to the identification of multiple mechanisms that may play a key role in regulating distinct aspects of anti-inflammatory response observed during asthma. The role of mast-cell-derived heparin in regulating physiological and pathophysiological responses is yet to be fully elucidated, however it has been suggested that the release of heparin after the degranulation of mast cells may possess a protective or regulatory role to limit the extent of subsequent inflammation. In experimental models, heparin was found to inhibit degranulation of mast cells and subsequent release of inflammatory mediators via inhibition of inositol triphosphate signalling. Heparin was also shown to bind directly to adhesion molecules like selectins which are involved in the process of inflammation. It is via NH_2-terminal lectin domain that carbohydrates calcium-dependently bind to these molecules on the surface of interacting cells and hence, limiting inflammatory cell recruitment during inflammation. Our *in-vitro* and *in-vivo* studies provide an insight on the non-anticoagulant effects that have been ascribed for enoxaparin. The non-anticoagulant molecules of enoxaparin were obtained after separation of entire polysaccharide using ion-exchange chromatographic technique. Two non-anticoagulant fractions of enoxaparin were identified that significantly inhibited peripheral blood mononuclear cell activation. A di- and a tetra-saccharide fraction of enoxaparin was shown to inhibit the release of inflammatory mediators

like interleukin (IL)-4, IL-5, IL-13 and tumor necrosis factor-α. The data also suggest that the observed response is likely to be due to an interaction of 6-*O*-sulfated tetrasaccharide with cellular receptor(s). Similarly, in other experimental model, IC-derived disaccharide of enoxaparin was found to significantly inhibit the release of other potential inflammatory markers like IL-6 and IL-8 from human pulmonary epithelial cells. The observed suppression of IL-6 and IL-8 by disaccharide was likely due to the inhibition of the activation of protease activated receptors on lung epithelial cell surface. The data from this study also demonstrated the importance of 6-*O*-sulfate groups to retain the observed anti-inflammatory activity of the non-anticoagulant enoxaparin fraction.

In animal models, heparin has been reported to confer benefits in allergen induced airway hyper-response or platelet-activating factor induced bronchial hyperresponsiveness. In one such reports prevention of nitric oxide signalling by heparin was reported to have benefit in methacholine-induced airway hyperresponsiveness. In addition to having effects in experimental settings, heparin has also shown potential in the management of human asthma in several controlled clinical studies.

It is evident from basic science investigations to clinical observations that heparin possess considerable promise for the management of asthma. It is reasonable to anticipate major advances in this field and will be fascinating to watch the future developments over the next few years as new anti-asthmatic agents that match the criteria required for a drug.

FUTURE DIRECTIONS

Over the past few decades a lot of effort has been put into finding novel therapeutic targets for effective management of asthma. Many novel treatments are specific, targeting a single mediator or receptor. Absolute specificity to one protein, as seen with monoclonal antibodies is not the most effective way to treat complex disorders like asthma; one pathway may be blocked, however complexity of the disease is such that symptoms are not alleviated. Drugs with more widespread effects as seen with non-anticoagulant molecules of heparin might prove to be more effective alternative and at a more favourable cost.

So far, continuous research have managed to concatenate many strings together and resolve many factors in this field including the identification of non-anticoagulant molecules within heparin which are responsible for its anti-inflammatory effects and the impetus for the development of advanced analytical techniques to dissociate non-anticoagulant molecules from the parent mixture. However, much still remains to be learned, the complexity of these macromolecules greatly complicate the detailed structure-activity relationships and the areas which require further investigation include: i) interpretation of fine/specific non-anticoagulant sequences and their precise structural information (including the role of specific sulphate groups) to enable better understanding of structure-activity relationships from the point of view of answering biological questions and to provide new targets for therapeutic interventions in different inflammatory disease states and ii) the mechanisms that regulate the observed anti-inflammatory effects of various non-anticoagulant sequences of heparins. Emergence of understanding such exact structural requirements and mechanisms may help to develop myriad of structurally

different non-anticoagulant preparations which seems to be the best way forward to correlate these preparations to various proteins and respective biological activities with a goal to maximise the therapeutic benefit for the disease being targeted. It is also increasingly important to conduct complete characterisation of all structural elements within heparin that account for its anti-inflammatory response since heparin preparations are currently standardised for its use as an anticoagulant and there are possibilities that certain features that account for their anti-inflammatory effects may be lost or selected out while processing raw heparin. Future research should also focus on a particular delivery route that would confine these non-anticoagulant molecules to the target organ; in case of respiratory disorders like asthma, inhaled route would be a safe and preferable route of administration. The current sophisticated analytical techniques and methodologies provide a basic platform to explain the mystery behind heparin and anti-inflammation however, it is by no means a trivial undertaking. With this knowledge, we may in the near future be able to influence further clinical trials and look forward to expand its use as an anti-asthmatic agent.

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